RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH.

Dissertation Submitted to

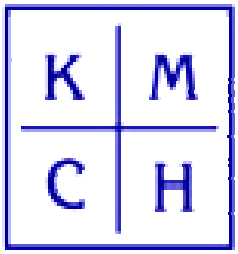
The TamilNadu Dr. M.G.R. Medical University, Chennai.

In partial fulfilment for the award of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

APRIL-2012



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

KMCH COLLEGE OF PHARMACY,

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COIMBATORE -641048.

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Under the guidance of

Prof.J.Dharuman M. Pharm, (Ph.D.)

Head of the Department

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This is to certify that, the work embodied in the thesis entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH"** *is a bonafide research work carried out by* ***Mr.T.M*anavalan (Reg. No: 26107225)**, *Student in Master of Pharmacy, Department of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, under the guidance of* **Prof. J. Dharuman, M.Pharm, (Ph.D)** *Head, Dept of Pharmaceutical Analysis, K.M.C.H. College of Pharmacy during the academic year 2011-2012.*

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I wish him best career,

Signature,

Date: **Prof. J. Dharuman, M. Pharm (Ph.D)**

Place:

DECLARATION

I am here by stating that, to the best of my knowledge and belief, the project report entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH** is being submitted for the partial fulfilment of **Master of Pharmacy in Pharmaceutical Analysis** for the academic year 2011-2012 of KMCH. College of Pharmacy affiliated to The Tamilnadu Dr. M.G.R. Medical University carried out under the guidance of **Prof. J. Dharuman, M.Pharm (Ph.D),** Head, Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore.

I abide that all the data presented in this report will be treated with utmost confidentiality.

Date: **T.Manavalan**

Place: **(Reg No: 26107225)**

EVALUATION CERTIFICATE

This is to certify that, the work embodied in the thesis entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH** submitted by **Mr.T.Manavalan (Reg. No: 26107225),** to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfilment for the Degree of **Master of Pharmacy**, in **Pharmaceutical Analysis**, is a bonafide research work carried out by the candidate at K.M.C.H. College of Pharmacy, Coimbatore, Tamilnadu, the same was evaluated by us during academic year 2011-2012.

Examination Center: KMCH College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

ACKNOWLEDGEMENT

My dissertation entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH "** would not have been a feasible one without the grace of god almighty who gave me morale till the completion of my project.

I am extremely thankful to my Academic Guide **Prof. J. Dharuman, M.Pharm, (Ph.D)** Head, Department of Pharmaceutical Analysis, KMCH. College of Pharmacy, for his constant insight, guidance, countless serenity, encouragement and painstaking efforts in my project work. I am indebted to his kindness and never failing cooperation.

To begin with I would like to thank **Dr. A. Rajasekaran**, **M. Pharm**, **Ph.D., Principal**, K.M.C.H. College of Pharmacy for his constant encouragement, support and the facilities provided.

I will always remain indebted to **Dr Nalla G. Palanisamy**, Chairman, and **Dr. Thavamani D. Palanisamy**, Managing Trustee, K.M.C.H. College of Pharmacy, Coimbatore for all the facilities, which have been provided to us at the institution, enabling me to do work of this magnitude.

My special thanks to all teaching and non-teaching staff members of KMCH College Pharmacy, Coimbatore, Library and computer lab faculties who directly or indirectly gave a helping hand during the course of study.

This project would not be a resplendent one without the timely help and continuous support by my ever Friends of the Pharmaceutical Ananlysis (**CH. Rambabu, M. Venkana Babu, T. Srikanth Reddy, J. K. Ghaharin, Dona Sara Kurian, A. V. S. Hemanth, Tinu Thomas, G. Arun Kumar)** and my M.Pharm Ist year juniors **(G. Venkatesh, T. Chaitanya,)** and I take this opportunity to acknowledge them with thanks.

Finally I would like to express my sincere thanks to all those people who directly or indirectly helped me to complete this work successfully.

Above all I dedicate myself before the unfailing presence of **GOD** and constant love and encouragement given to me by my beloved **Father, Mother, Sister** and all of my family members who deserves the credit of success in whatever work I did.

Yours sincerely,

T.Manavalan.

ABBREVIATIONS

HPLC	*High Performance Liquid Chromatography*
UV	*Ultra violet*
BA	*Bioavailability*
M.W.	*Molecular weight*
e.g.	*Example*
i.e.	*That is*
%	*Percentage*
PDA	*Photo Diode Array*
I.S	*Internal Standard*
ACN	*Acetonitrile*
CDSCO	*Central Drugs Standard Control Organization*
RF	*Response Factor*
Mg	*Milligram*
Ml	*Milliliter*
µg	*Microgram*
W/w	*Weight by weight*
V/v	*Volume by volume*
µg/ml	*Microgram per milliliter*

ng /ml	*nanogram per milliliter*
PH	*Hydrogen ion concentration*
^{0}C	*Degree centigrade*
T	*Time*
Abs.	*Absorbance*
Conc.	*Concentration*
Fig.	*Figure*
Tab.	*Table*
M.P.	*Melting Point*
AUC	*Area under curve*
CV	*Coefficient of variance*
RSD	*Relative standard deviation*
L/h	*Liter per hour*
L/kg	*Liter per kilogram*
Rpm	*Rotation per minute*

INDEX

INTRODUCTION

Nature has given on us a very rich botanical wealth and various types of plants grow in different parts of the country (Jignaet al., 2005). According to a conservative estimation, 300,000-400,000 plant species grow on Earth; however, only a small percentage have had their phytochemistry and biological function investigated (Kitani, et al., 2001). Since ancient times herbal medicine are used for various treatment and in most of the developing countries 75-80% of the whole population rely on herbal medicine because of better cultural acceptability, better compatibility with the human body and fewer side effects. Hence, the last few years have seen a major increase in their use in the developed countries. For example four out of ten Americans used alternative medicine therapies in 1997; total visits to alternative medicine practitioners increased by almost 50% from 1990 and exceeded the visits to all US primary care physicians (Grabley and Thiericke, 1999). Thirty per cent of the worldwide sales of drugs are based on natural products. India is rich in different levels of biodiversity, namely species diversity, genetic diversity and habitat diversity. In India enormous number of medicinal plants are available naturally which accounts for great economic values (Jignaet al., 2005).

The Indian system of medicine (Ayurveda, Siddha) and Chinese system of medicine are two of the most extensively developed medicinal system in the world. Since ancient times Ayurveda remains one of the most and yet living traditions practised widely in India, Sri Lanka and other countries and has a sound philosophical and experiential basis (Dahanukaret al., 2000; Chopra and Doiphode, 2002). Indian healthcare consists of medical pluralism and Ayurveda still remains dominant compared to allopathic medicine, especially for treatment of a variety of chronic disease conditions (Waxler, 1998). Total global herbal drug market including herbal products and raw material is estimated as US $62 billion with the annual growth rate of 5-15% and is expected to grow US $5 trillion by the year 2050. Ayurveda contributes Rs 3500 crores (US $813 million) annually to the internal market. The Indian medicinal plants-based industry is growing at the rate of 7–15% annually. The value of medicinal plants-related trade in India is estimated at Rs 5000 crores per annum. Global trend leading to increased demand of medicinal plants for pharmaceuticals, phytochemicals, nutraceuticals, cosmetics and other products is an opportunity sector for Indian trade and commerce (Singh et al., 2003). Herbal preparations and phytomedicines are increasingly being used by the public as self-selected OTC products for therapeutic or preventative

purposes. Hence, pharmaceutical sector is focused on development of new drugs, innovative/indigenous processes for known drugs and development of plant-based drugs through investigation of leads from the traditional systems of medicine. Even though the current accepted modern medicine or Allopathy has gradually developed over the years by scientific and observational efforts of scientists, the basis of its development remains rooted in traditional medicine and therapies. Ancient wisdom has been the basis of modern medicine and will remain as one important source of future medicine and therapeutics (Patwardhan and Hooper, 1992).

Nature based treatments gaining much importance now a days due to number of reasons like development of drug-resistance by microorganisms to antibiotics, side effects of modern drugs, and emerging diseases where no medicines are available, which have stimulated the interest on plants as a significant source of new medicines. Researchers are experiencing difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity. A number of synthetic drugs have adverse and unacceptable side effects. There have been impressive successes with botanical medicines. Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on (Dahanukaret al., 2000) to bring the numerous molecules like rauwolfia alkaloids for hypertension, psoralens in vitiligo, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, mucunapruriensfor Parkinson's disease, piperidines as bioavailability enhancers and baccosides in mental retention (Patwardhan, 2003). A whole range of chronic and difficult-to-treat diseases such as cancers, cardiovascular disease, diabetes, rheumatism and AIDS, all require new effective drugs. Therefore, pharmaceutical industry is now focused to develop herbal based medicines. As a result many herbal products are available in market but the quality, efficacy and safety remain unclear. Because herbal products are easily adulterated, mislabelled and even lacking in active content. Hence, standardization is an important issue and this seems to be a logical solution for quality control of herbal products, however it is not an easy process due to the presence of complex phytoconstituents.

Most of the standard setting organisations like Indian Pharmacopoeia, European Pharmacopoeia and British Pharmacopoeia contain important requirements pertaining to certain analytical procedures and acceptance criteria that are relevant to herbal drugs, herbal preparations and their formulations for quality control and standardization. Validated analytical procedures are required for the quantification of active compounds present in

herbal medicinal preparations. For these, chromatographic methods like high performance liquid chromatography (HPLC), thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), and gas chromatography (GC) are routinely used as valuable tools for qualitative determination of small amounts of impurities, Identification and quantification of active constituent present in the herbal formulations. (Quality Control Methods for Medicinal Plant Materials, WHO, Geneva, 1998).

High-performance liquid chromatography (HPLC) is nowadays one of the separation techniques most extensively used in the phytochemistry and pharmaceutical industry for quality control because of its high selectivity, efficacy and speed (Merken and Beecher, 2000). Validation of an HPLC test method is mandatory in implementing a quality control system in any laboratory and ensures that the methodology is sufficiently selective, accurate, reproducible and robust over the range specified for analysis (ICH, 1996)

The present study is aimed to develop a validated HPLC method for the quantification of All-trans retinoic acid in plant extracts namely Broccoli (**Brassica oleracea),** Spinach **(Spinacia oleracea).**

Analytical methods development and validation [1-4] plays a crucial role in the discovery, development, and manufacture of pharmaceuticals.

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product.

These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. High performance liquid chromatography (HPLC) for drug products containing more than one active ingredient. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

The drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and

introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Importance of analytical chemistry is to gain information about the qualitative and quantitative composition of substance and chemical species, that is, to find out what a substance is composed of and exactly how much it is present.

Pharmaceutical analysis deals with the analysis of the pharmaceutical substances. It is generally known that pharmaceutical is a chemical entity of therapeutic interest.

Pharmaceutical analysts in research and development (R&D) of pharma industry plays a vital role in new drug development and follow up activities to assure that, a new drug product meets the established standards, its stability, and continued to meet the purported quality throughout its shelf life.

Analytical method development is required for:

- Herbal products
- New process and reactions
- Active ingredients (Macro analysis)
- Residues (Microanalysis)
- Impurity profiling
- Component of interest in different matrices
- Clinical pharmacokinetic studies
- Quality control department in industries

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantitation of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,

- ❖ The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

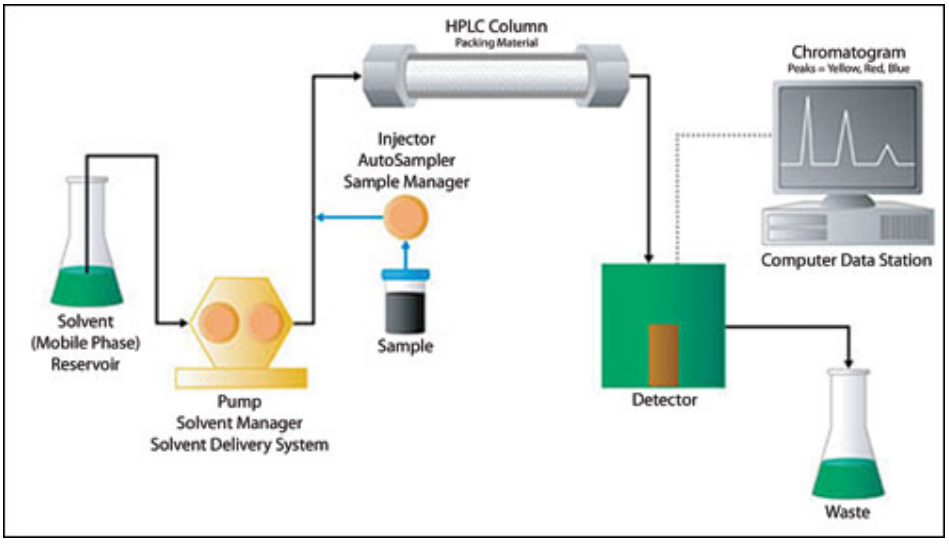
Method development [5-7]

- Planning
- Method development
- Customer evaluation testing
- Validation experiments
- Method transfer experiments
- Final method in use
- Final result

CHROMATOGRAPHY [8, 9]

Chromatography is a group of technique for the separation of the compounds of mixtures by their continuous distribution between two phases. One is stationary phase and the other is mobile phase. As a general rule, highly polar materials are best separated using partition chromatography, while very non polar materials are separated using adsorption chromatography. Between extremes, either process might be applicable.

Fig: 1 Schematic Diagram of a HPLC instrument



HPLC Methods of Analysis for Drugs in Combination:

Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases),
- Reusable columns (expensive columns but can be used for many analysis),
- Ideal for the substances of low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation tends itself to automation and quantitation (less time and less labour),
- Precise and reproducible,
- Calculations are done by integrator itself,
- Suitable for preparative liquid chromatography on a much larger scale.

The different types of chromatography

- ✓ Adsorption chromatography
- ✓ Partition chromatography
- ✓ Ion Exchange chromatography
- ✓ Gel permeation chromatography
- ✓ Column chromatography

Table no: 1 CLASSIFICATION OF COLUMN CHROMATOGRAPHIC METHODS

General classification	Specific method	Stationary phase	Type of equilibrium
Liquid Chromatography (LC) (mobile phase: liquid)	Liquid-Liquid or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-Bonded Phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-Solid or Adsorption	Solid	Adsorption
	Ion exchange size exclusion	Ion-exchange resin Liquid in interstices of a polymeric solid	Ion exchange Partition/sieving
Gas Chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded Phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-Solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: Super critical fluid		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography.

In the normal phase mode, the stationary phase is polar (bonded siloxane with a polar functional group-polarity order: cyano<diol< amino <dimethylamino) and the mobile phase is nonpolar (n-hexane, methylene chloride, or chloroform) in nature. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These

compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non-polar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel and the mobile phase is polar solvent (methanol, acetonitrile, tetrahydrofuran, water, or usually a mixture of water with one of the organic solvents).

An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity and for adjusting the polarity. The organic solvent is called the **modifier**, and acetonitrile is the most common one.

The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octadecylsilane (ODS) or C_{18}, $C_{8,}$ C_4, etc., (in the order of increasing polarity of the stationary phase).

Reverse phase chromatography is widely in use due to the following advantages.

- Many compounds such as biologically active substances, have limited solubility in the nonpolar mobile phase that are employed in normal-phase chromatography.
- Ionic or highly polar compounds have high rate of adsorption on straight silica or alumina columns and therefore can elute as tailing peaks.
- Column deactivation from polar modifiers is a problem in liquid-solid chromatography which frequently can lead to irreproducibility in chromatographic systems.
- Long re-equilibration times during gradient elution are common in adsorption chromatography. The use of reversed phase chromatography with bonded-phase columns is advantageous because of the short re-equilibration time required.

- Ionic compounds can be chromatographed by using ion-exchange chromatography. This mode of chromatography is tedious because precise control of variables such as pH and ionic strength is required for reproducible chromatography.

QUANTITATION:

Quantitation Methods in HPLC:

Peak height or peak area measurements only provide a response in terms of detector signal. This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for quantitation are

1. Normalized peak area method
2. External Standard method
3. Internal Standard method
4. Method of Standard addition

1. Normalized peak area method:

The area percent of any individual peak is referred to the normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method; the response factor for each component is identified.

2. External Standard method:

This method includes injection of both standard and unknown and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (R_f) can be determined for each standard as follows

$$R_f = \frac{\text{Standard Area (Peak height)}}{\text{Standard Concentration}}$$

The external standard approach is preferred for most samples in HPLC that do not require extensive sample preparation. For good quantitation using external standards, the chromatographic conditions must remain constant during the separation of all standards and

samples. External standards are often used to ensure that the total chromatographic system is performing properly and can provide reliable results.

3. Internal Standard method:

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of the internal standard. The accuracy of this approach obviously dependence on the structural equivalence of the compounds of interest and the internal standard.

The requirements for an internal standard are:

- It must have a completely resolved peak with no interferences,
- It must elute close to the compound of interest,
- It must behave equivalent to the compound of interest for analysis like pretreatments, derivative formations, etc.
- It must be added at a concentration that will produce a peak area or peak height ratio of about unity with the compound,
- It must not be present in the original sample,
- It must be stable, un reactive with sample components, column packing and the mobile phase and
- It is desirable that this compound is commercially available in high purity.
- The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. To be able to recalculate the concentration of a sample component in the original sample, we have to demonstrate first the response factor. The response factor (RF) is the ratio of peak areas of sample component (A_x) and the internal standard (A_{ISTD}) obtained by injecting the same quantity. It can be calculated by using the formula,

$$Rf = \frac{Ax}{A.I.STD}$$

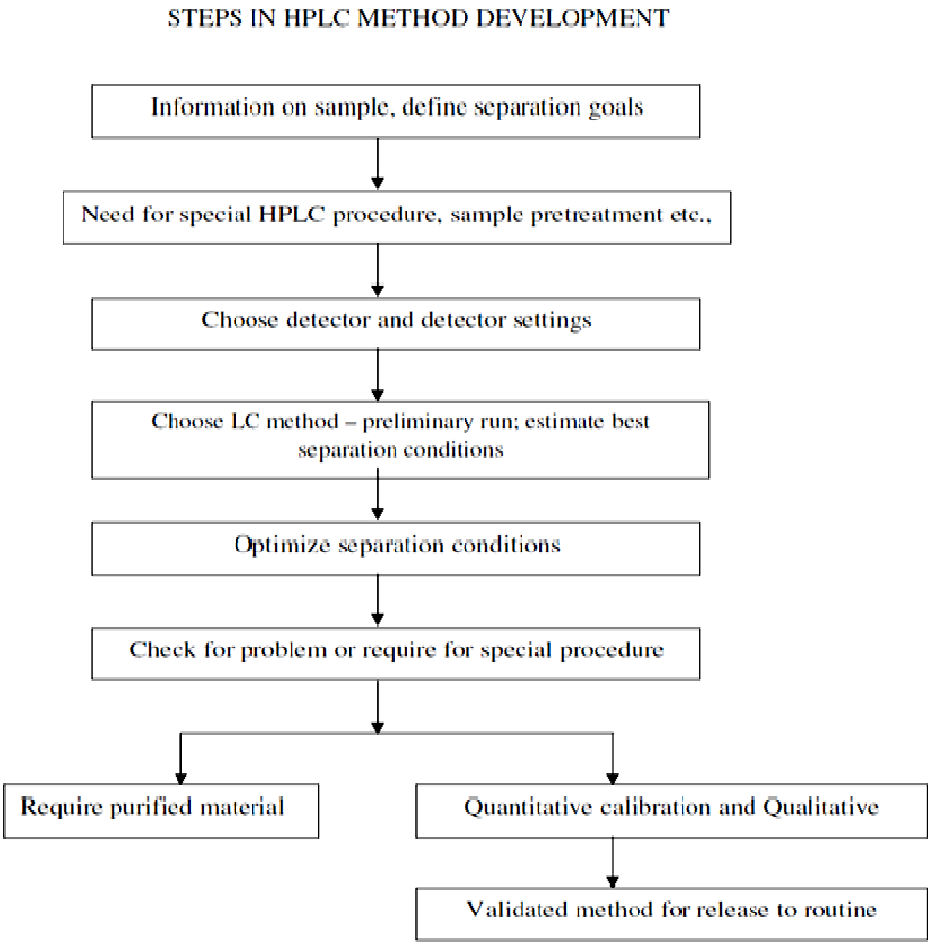
On the basis of the response factor and strength of the internal standard (N_{ISTD}), the amount of the analyte in the original sample can be calculated using the formula,

$$X = \frac{A_S}{R_F . A_{1STD}} x N_{1.STD}$$

The calculations described above can be used after proving the linearity of the calibration curve for the internal standard and the analytical reference standard of the compound of interest. When more than one component is to be analyzed from the sample, the response factor of each component should be determined in the calculations using similar formula.

4. Method of Standard addition:

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is most often used in trace analysis. An important aspect of the method of standard addition is that the response prior to spiking additional analytes should be high enough to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision.



Flow chart of HPLC method development

Optimization of Chromatographic Conditions:

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that all the compounds are detected by more or less symmetrical peaks on the chromatogram. By a slight change of the mobile phase composition, the shifting of the peaks can be expected.

From a few experimental measurements, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram is the one in which all the peaks are symmetrical and are well separated in less run time.

System Suitability:

System suitability testing is an integral part of analytical procedures.

The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

The peak resolution can be increased by using a more efficient column with higher theoretical plate number, N.

The parameters that are affected by the changes in chromatographic conditions, It includes the following parameters, but not necessarily to comply all the parameters.

- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

i) Resolution (R_s)

The resolution, R_s, of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

$$R_s = \frac{Rt_2 - Rt_1}{0.5(W_1 + W_2)}$$

Where, Rt_1 and Rt_2 are the retention times of components 1 and 2 and

W_1 and W_2 are peak widths of components 1 and 2.

ii) Capacity factor (k')

Capacity factor, k', is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. Capacity factor is a measure of how well the sample molecule is retained by a column or TLC plate during an

isocratic separation. The ideal value of k' ranges from 2-10. Capacity factor can be determined by using the formula,

$$K' = \frac{V_1 - V_0}{V_0} \times S$$

Where, V_1 = retention volume at the apex of the peak (solute) and

V_0 = void volume of the system.

The values of k' of individual band increase or decrease with changes in solvent strength. In reversed phase HPLC, solvent strength increases with the increase in the volume of organic phase in the water / organic mobile phase. Typically an increase in percentage of the organic phase by 10 % by volume will decrease k' of the bands by a factor of 2-3.

iii) Selectivity (α)

The selectivity (or separation factor), α is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the formula,

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak respectively.

iv) Column efficiency (N)

Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Smaller the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 2000 - 100,000 plates/meter are ideal for a good system. Efficiency is calculated by using the formula,

$$N = 16\frac{Rt^2}{W^2},$$

Where, R_t is the retention time and W is the peak width.

v) Peak asymmetry factor (As)

Peak asymmetry factor, as, can be used as a criterion of column performance. The peak half width, b, of a peak at 10 % of the peak height, divided by the corresponding front half width, a, gives the asymmetry factor.

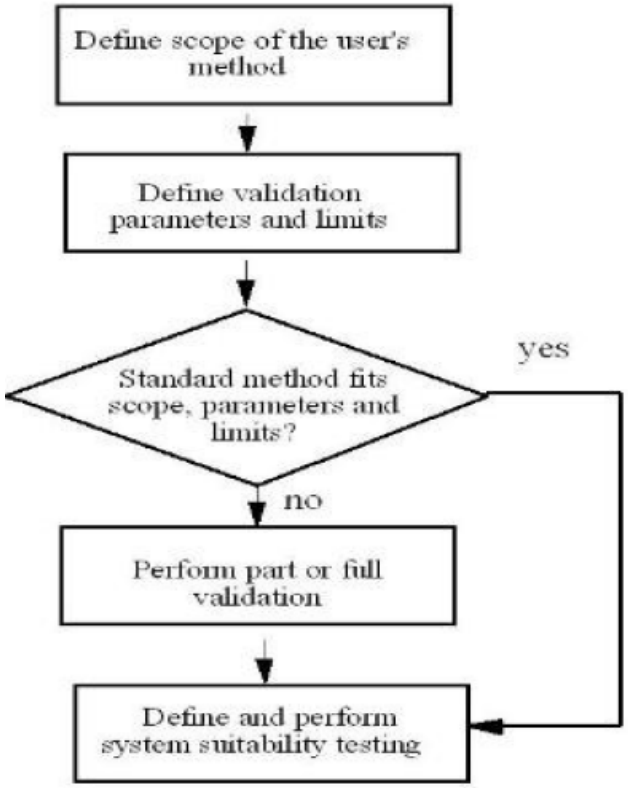
$$As = \frac{b}{a}$$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

System Suitability Parameters and Recommendations (ICH Guidelines)

Parameter	**Recommendation**
Capacity Factor (k')	the peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Relative retention	not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.
Tailing Factor (T)	T of </= 2
Theoretical Plates (N)	In general should be > 2000

VALIDATION OF ANALYTICAL METHOD:



INTRODUCTION:

Analytical method validation is the process of demonstrating that analytical procedures are suitable for their intended use and provide accurate test results that evaluate a product against its defined specification and quality attributes [10].

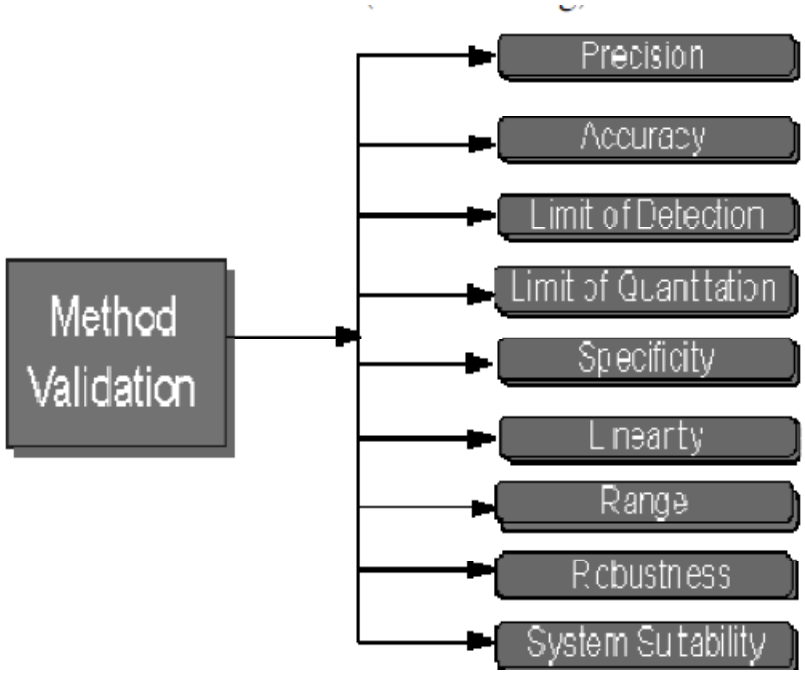
The U.S. Federal Register states "Validation data must be available to establish that the analytical procedures used in testing meets proper standards of accuracy and reliability [11]"any analytical test methods are expected to be used in a Quality Control environment they require an additional degree of refinement compared to research methods **[12].**

The following observation will explain the relationship between validation and method development.

- When methods are properly developed, they readily validate.
- Validation is not a method development tool and it does not make a method good or efficient.
- Validation acceptance criteria should be based on method development experience.

VALIDATION OF ANALYTICAL PROCEDURES [13-17]

Different Types of Validation characteristics [18]



Generalized validation process for an HPLC assay method:

Validation is the process of collecting documented evidence that the method performs according to its intended purpose.

1. Precision:

The closeness of agreement between a series of measurements multiple samplings of the same homogeneous sample under prescribed condition.

The precision of test method is usually expressed as the standard deviation or relative standard deviation of a series of measurements.

Precision may be considered at three levels: Repeatability, Intermediate Precision and Reproducibility.

Acceptance Criteria:

- Percentage Relative standard deviation (%RSD) NMT 1 % (Instrument precision)
- (%RSD) NMT -2% (Intra- assay precision)

2. Accuracy [19]:

The ICH guideline recommends that accuracy should be determined using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (ICH, 1996). Spiked samples are prepared in triplicate at three levels over a range that covers 80 -120% of the target concentration for assay methods and over a range that covers the expected impurity content of a sample for impurity methods (Shabir, 2003).

There are several methods that can be used for determining accuracy. The most common include:

Analyze a sample of known concentration and compare the measurement to the true value. In this case, method accuracy is the agreement between the difference in the measured analyte concentration and the known amount of analyte added. That is the accuracy or % recovered is calculated as:

$$\frac{C_m \times 100}{C_t}$$

Where C_m is the measured concentration and C_t is the theoretical concentration.

Accuracy has also been reported as a sample is analyzed and the measured value should ideally be identical to the true value. Accuracy is represented and determined by recovery experiments. The usual range is being 10% above or below the expected range of claim. The % recovery was calculated using the formula,

$$\%\,\mathrm{Recovery} = \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

Acceptance Criteria:

- For an assay method, mean recovery will be 100%± 2% at each concentration over the range of 80-120% of the target concentration.
- For an impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities in the range of 0.1-2.5 % (V/W).

3. Detection Limit:

It is lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.

Following are different approaches:

i. **Visual Evaluation Method:**

Prepare the sample solutions with known lowest possible concentrations of analyte and establish the minimum concentration at which the analyte can be reliably detected by analyzing as per test method.

ii. Based on Signal to Noise Ratio Method:

The LOD can be expressed as a concentration at specified signal-to-noise ratio obtained from samples spiked with analyte. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable.

iii. Based on the standard Deviation of the Response and the Slope:

- Prepare the blank solution as per test method and inject six times into the chromatographic system.
- Similarly prepare the linearity solution staring from lowest possible concentration of analyte to 150 % (or as per protocol) of target concentration and establish the linearity curve.

The detection limit (DL) may be expressed as:

$$LOD = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

4. Quantitation Limit:

It is lowest amount of analyte in a sample, which can be quantitatively determined with acceptable accuracy and precision.

Following are different approaches:

i. **Visual Evaluation Method:**

Prepare the sample solutions with known lowest possible concentrations of analyte and establish the minimum concentration at which the analyte can be reliably quantified by analyzing as per test method.

ii. **Based on signal to noise ratio method :**

The LOQ can be expressed as a concentration at specified signal-to-noise ratio obtained from samples spiked with analyte. A signal-to-noise ratio of 10:1 is generally considered acceptable. The ratio recognized by the ICH (1996) is a general rule. It has been stated that "The determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases".

iii. **Based on the standard Deviation of the Response and the Slope:**

- Prepare the blank solution as per test method and inject six times into the chromatographic system.
- Similarly prepare the linearity solution staring from lowest possible concentration of analyte to 150% (or as per protocol) of target concentration and establish the linearity curve.

The Quantification limit (QL) may be expressed as:

$$LOQ = \frac{10 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

Perform the Precision and accuracy at the level of limit of quantification by spiking LOQ concentration on placebo / Drug product / Drug substance.

For detail methodology and acceptance criteria refer Precision and accuracy of test method.

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.
- LOQ defined as the lowest concentration providing a RSD of 5%.

LOQ should be at least 10% of the minimum effective concentration for clinical applications

5. Specificity:

The ability to assess unequivocally the analyte in the presence of components that may be expected to present, such as impurities, degradation products and matrix components, etc.

Specificity shall be demonstrated by performing Placebo / blank interference and forced degradation studies.

1. **Blank interference:**

Prepare blank solution as per test method and analyse as per test method.

2. **Placebo interference (In case of Drug products):**

Prepare the placebo solution equivalent to the test concentration (Subtract the weight of active ingredient) and analyse as per test method.

3. **Force Degradation studies:**

Degrade the sample forcefully under the various stress conditionslike Light, heat, humidity, acid / base / water hydrolysis and oxidation and ensure the degradation from 1 % to 20 %.

- **Light:** Expose the Drug product, drug substance and placebo to UV light for about 200 watt hours / square meter and the overall illumination not less than 1.2 million Lu hours [17] for visible light. Prepare the sample and placebo solution as per test method and analyse.
- **Heat:** Expose the Drug product, drug substance and placebo at 105 °C for about 12 hours (For substance having low melting point below 10°C of its melting point). Prepare the sample and placebo solution as per test method and analyse.
- **Humidity:** Expose the Drug product, drug substance and placebo for about 80 % RH at about 25°C for about one week. Prepare the sample and placebo solution as per test method and analyse.
- **Acid / Base:** Prepare the acid or base solution of 0.1N and reflux the sample and placebo with 50 ml of acid / base solution for about 1 hour at 60°C. Neutralize the solution and dissolve the contents in diluents as per test method. Change the strength of acid and base or reflux time to ensure the desired degradation.

- **Water:** Reflux the sample / placebo with 100 ml of purified water for 12 hour at 60°C. Dissolve the contents in diluents as per test method. Change the reflux time so as to ensure the desired degradation.
- **Oxidation:** Reflux for 12 hour at 60°C with 1 % H_2O_2 or suitable oxidant. Dissolve the contents in diluents as per test method. Change the reflux time so as to ensure the desired degradation.

Note: Based on the physicochemical properties and literature stress conditions can be decided.

Acceptance Criteria:

- Placebo / Blank should not elute at the retention time of analyte peak and known impurity peak.
- Peak purity of analyte peak should be confirmed.
- Degradation of active analyte peak should be from 1% to 20%.

6. **Linearity and range:**

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) proportional to the analyte concentration in samples within a given range. The linear range of detectability that obeys Beer's law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation,

$$Y= aX + b$$

together with the correlation coefficient of determination r. For the method to be linear the r value should be close to1. Where **Y** is the measured output signal, **X** is the concentration of sample, **a** is the slop, b is the intercept.

The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

If linearity is not meeting the acceptance criteria, establish the range of concentration in which it is linear.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

7. Robustness:

It is a measure of method's ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

For example a chromatographic method, the typical method parameters need to change deliberately and verify during method validation:

Flow rate	:	(+/- 0.2ml/minutes).
Mobile phase composition	:	(+/- 10% of organic phase).
Column oven temperature	:	(+/- 5°C).
PH of buffer in mobile phase	:	(+/- 0.2 units).
Filter suitability	:	(At least two filters).

For Variations:

1. System suitability should meet the acceptance criteria as per test method.
2. If system suitability doesn't meet, narrow the variation range and carryout the experiment again to meet system suitability.

8. Ruggedness:

The United States of Pharmacopeia (USP) defines Ruggedness as "the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, and different lots of reagents. Ruggedness is a measure of Reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst".

The following are the typical method parameters need to be tested during method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability.
- System-to-System variability.
- Different days.
- Different Laboratories.
- Stability of Solutions and mobile phase. (At least for 48 hours)

Method Validation Requirements for Example (ICH)

Table no: 2

Method Validation requirements	Acceptance Criteria
Precision	
Assay repeatability	≤ 1% RSD
Intermediate precision (Ruggedness)	≤ 2% RSD
Accuracy	
Mean recovery per concentration	100.0% ± 2.0%
Limit of detection	
Signal to-to-noise ratio	≥ 3:1
Limit of quantification	
Signal to-to-noise ratio	≥ 10:1
Linearity/Range	
Correlation coefficient	>0.99
y-Intercept	± 10%
Visual	Linear
Robustness	
System suitability met	yes
Solution stability	± 2% change from time zero
Specificity	
Resolution from main peak	>2 min. (retention time)

REVIEW OF LITERATURE

Vickie Tatum et al(2005) [20] have reported RP- HPLC assay method for All-trans retinoic acid. Chromatographic separation was achieved on an C_{18} reversed phase column Using 85% methanol and 15% 0.01 M sodium acetate buffer, pH 5.2, as the mobile phase and at a flow rate of 1.5 ml/min. Retinol, retinal, all-trans-retinoic acid and 13-cis-retinoic acid were eluted from a 5 μm C18 reversed-phase column (4.6mm I.D., 15 cm) in less than 14 min. Retinyl acetate, was eluted in 25.5 min under the above conditions. Each compound was detected at the most sensitive wavelength by coupling fluorescence and UV detection.

Lopez-Abater et al(2003) [21] have developed a method for the simultaneous determination of retinol acetate, α-Tocopherol and α-Tocopherol acetate. Method I: the fat milk sample was extracted with ethanol-hexane and injected directly into the chromatographic column. Method II: the power milk sample was extracted with ethanol-hexane and also injected directly into the column. Under optimum conditions the limits of detection for retinol acetate, δ-, β-, α-Tocopherol and α-Tocopherol acetate were 0.33, 21.2, 32.9, 32.5 and 3 .2 ng and the limits of quantification were 0.42, 25.3, 37.9, 36.8 and 6.3 ng, respectively. The precision results showed that the relative standard deviations of repeatability and reproducibility were between 0.74 and 5.7%.

David J. Hart et al(1999) [22] have performed studies that examine the factors which affect the chromatographic response of carotenoids and contribute to analytical variation and inaccuracies in their quantitative determination. The addition of a solvent modifier (triethylamine) to the mobile phase was shown to improve the recovery of carotenoids from the column from around 60% to over 90%. Analysis showed that good sources (>1000 μg/100 g) of lutein were broccoli, butter head lettuce, parsley, peas, peppers, spinach and watercress; of lycopene: tomatoes and tomato products; and of β-carotene: broccoli, carrots, greens, butter head lettuce, mixed vegetables, parsley, spinach and watercress. There was little or no loss of carotenoids on cooking, green vegetables showed an average increase in lutein levels of 24% and in β-carotene levels of 38%.

Erin M. Siegel et al(2001) [23] have been reported that retinoids have cancer chemotherapeutic and chemopreventive activities. Total RA concentration and the concentrations of RA isomers (all-trans-RA, 13-cis-RA, and 9-cis-RA) were measured by high-pressure liquid chromatography in serum samples. The relative abundance of the three

RA isomers was similar for each visit with 13-cis-RA having the highest concentrations followed by 9-cis-RA and all-trans-RA. The within-person variability of total RA and individual isomers was low.

Raphael Vazquez et al(2009) [24] have reported a RP-HPLC separation on an Zorbax C18 column (150 mm x 4.6 mm, 2.5 µm particle size)using a mobile phase consisting of a mixture ammonium acetate 10 mM, pH 4.5 (solvent A) and methanol (solvent B). The separation was achieved within 35 minutes and the detection was performed by UV spectrometry-tandem mass spectrometry. The validation of the method was performed by the statistical evaluation for intra-day (n =6) and inter-daycalibrations (n =7); it was found to be satisfactory in terms of selectivity, accuracy (recovery between 100.0 and 102.3%) and precision (intra-day and inter-day precision between 1.0 and 10.2% and between 0.7 and 11.8%, respectively).

Amin Ismail et al(2003) [25] have developed a RP-HPLC method for vitamin analysis, to identify and quantify β -carotene, vitamin C and riboflavin. A ultra sphere octa decyl silyl (ODS) hypersil C18, 5 mm particle size, in a 250 mm length x 4.0 mm I.D stainless steel column is used with a mobile phase mixture of acetonitrile: methanol: ethyl acetate (88:10:2). Flow rate of 1ml/min is maintained. UV detection at detection at 250nm.

A.M dzerk et al(1998) [26] have been developed a RP-HPLC method for the quantitation of 9-cis-retinoic acid and its major metabolite, 4-oxo-9-cis-retinoic acid in human plasma. Samples were buffered and extracted with methyl-tert-butyl-ether. The analytes and an I.S. were separated on a C_{18} HPLC column using a shallow gradient of 70–89% organic solvent. The analytes were quantitated by UV detection at 348 nm. Selectivity against endogenous compounds and potential metabolites was demonstrated. The run time was 29 min. The standard curve was linear from 2.5 to 450 ng/ml. Inter assay precision for both analytes in quality control samples was less than 5.0% RSD.

Prachikabra et al(2003) [27] have developed a RP-HPLC method and validated for the determination of Isotretinoin in pharmaceutical dosage form. Chromatographic separation was carried out on a C-8 column using a mobile phase consisting of acetonitrile: isopropyl alcohol (50:50, v/v) adjusted at pH 5.0 using 1% ortho phosphoric acid. Flow rate was 1ml/ min and UV detection was carried at 280 nm. Caffeine was used as an internal standard. The calibration curve was linear over the range 5–600µgml−1. R.S.D. for precision study was

found to be <1%. The result of accuracy study was ranged between 98.61% and 101.51% with a R.S.D. lower than 2%. LOD and LOQ were found to be 0.0428µgml−1 and 0.1298µgml−1, respectively.

Lopez LB et al(2002) [28] have been developed a methodology for the quantification of vitamin A in human milk. Vitamin A levels were assessed in 223 samples corresponding to the 5th, 6th and 7th postpartum months. The samples were saponified with potassium hydroxide/ethanol, extracted with hexane, evaporated to dryness and reconstituted with methanol. A column RP-C18, a mobile phase methanol/water (91:9 v/v) and a fluorescence detector (lambda excitation 330 nm and lambda emission 470 nm) were used for the separation and quantification of vitamin A. The analytical parameters of linearity (r2: 0.9995), detection (0.010 microg/ml) and quantification (0.025 microg/ml) limits, precision of the method (relative standard deviation, RSD = 9.0% within a day and RSD = 8.9% among days) and accuracy (recovery = 83.8%) demonstrate that the developed method allows the quantification of vitamin A in an efficient way.

Prachikabra et al(2000) [29] have developed a simple, sensitive and specific UV spectrophotometric for the estimation of isotretinoin in bulk and soft gelatin capsule for routine analysis. The optimum conditions for analysis were established and validated in conformance with ICH guidelines. It was observed, the absorbance maximum (λmax) for Isotretinoin was 344 nm in methanol and the linearity was in the range of 1-8 µg/ml with coefficient of correlation as 0.9994. The lower limit of detection and the limit of quantification were found to be 0.2519 and 0.7634µg/ml respectively.

Stephen A. Barnett et al(1979) [30] have developed a RP-HPLC method for the simultaneous estimation of Vitamin A acetate Vitamin D_2 and Vitamin E acetate in multivitamin mineral tablets. The method requires dissolution of the sample in water-ethanol-pyridine solution (50:46:4), extraction of the vitamin in to warm hexane addition of cholesterol benzolate internally standard and separation with a methanol water gradient solution of coupled µBond pack phenyl-µbond pack column. Detection of the vitamin and internal standard is monitored at 280nm with separation accomplished in approximately in 15mn.

Clyde A. Collins et al(2001) [31] have developed a rapid and sensitive RP-HPLC method with fluorescence detection for the analysis for vitamin A and its acetate. The method

employs a C_{18} reversed-phase column and methanol as an eluent. The detection of these two compounds is monitored with fluorescence excitation at 348 nm and emission at 470 nm. Detector noise established the lower limit of quantitation at approximately 0.5 ng. Plasma samples were employed to evaluate the accuracy, reproducibility, and applicability of the method. Less than 1 ng of vitamin A in plasma can be quantitated by this procedure.

Zafar Iqbala et al(2001) [32] have developed a novel, simple and fast reversed-phase HPLC/UV method for simultaneous determination of all-trans-retinol and α-Tocopherol in human serum using Retinyl acetate as internal standard in the concentration of 0.5 μg/ml. A liquid-phase extraction was applied to the 250 μl of serum with n-hexane–dichloromethane mixture (70:30, v/v), in two steps, using ethanol–methanol mixture (95:5, v/v) for protein precipitation and BHT (butylated hydroxy toluene) as stabilizer for sample preparation. Both analytes were analyzed on Kromasil 100 C_{18} column (150 mm × 4.6 mm, 5 μm), protected by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge, at 292 nm wavelength, using methanol–water (99:1, v/v), in isocratic mode as mobile phase applied at flow rate of 1.5 ml/min and 1 ml/min for both 5 μm and 3 μm columns, respectively. Complete separation of all the analytes was achieved in 3 and 6 min on 3 μm and 5 μm columns.

Lorena Rigogaspar et al(2010) [33] have reported a simple RP-HPLC method to evaluate the influence of two currently available photostabilizers on cosmetic formulations containing combined UV-filters and vitamins A and E. UV-irradiated formulation samples were submitted to a procedure that included a reliable, precise and specific HPLC method employing a C18 column and detection at 325 and 235 nm. Methanol, isopropanol and water were the mobile phases in gradient elution. The method precision was between 0.28 and 5.07.

Benedicte Morina et al(2007)[34] have reported the effect of the fat-soluble vitamin A or vitamin E and grape seed proanthocyanidin extract (GSPE) on oxidative DNA damage estimated by 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG) contents in urine and leukocyte of rats. The aim of this study was to establish whether anthocyanidins could act as putative antioxidant micronutrients.

DRUG PROFILE

All-trans retinoic acid

IUPAC name **:** (2E,4E, 6E,8E)-3, 7-dimethyl-9-(2, 6, 6-trimethylcyclohexen-1-Yl) nona-2, 4,6,8-Tetraenoic acid 3, 4, 5-Trihydroxybenzoic acid, RA.

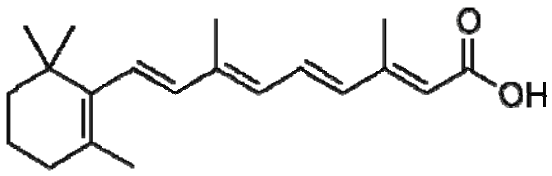
Synonyms **:** vitamin A acid; **Tretinoin** is the acid form of vitamin A and is also Known as **all-trans retinoic acid** or **ATRA**. It is a drug ATRA.

Molecular weight **:** 300.4412 g/mol.

Chemical formula **:** $C_{20}H_{28}O_2$.

STRUCTURE

Fig 2: Chemical structure of All-trans retinoic acid



Physical and chemical properties:

Appearance : Yellow to light orange crystalline powder.

Odour : Characteristic floral odour.

Solubility : It is practically insoluble in water; slightly soluble in alcohol and chloroform, sparingly soluble in ether, and soluble in methanol. ATRA is soluble at about 40 mg/ml in DMSO and at about 2.7 mg/ml in 95% ethanol fat and non-polar solvents.

Melting point : 180-182 °C, crystals from ethanol.

Storage : ATRA is more sensitive to light, heat, and air in solution.

Category : Commonly used to treat acne vulgaris and keratosis pilaris. It is also used to treat acute promyelocytic leukaemia (APL).

PLANT PROFILE-1

Description of plant: Broccoli

Botanical information:

Botanical name : Brassica oleracea.

Family : Brassicaceae

Fig: 3 Brassica oleracea

Common names : Broccoli, brocks, calabrese, Italian asparagus, Italian broccoli

Vernacular names:

Hindi : Hari phulagobi

Tamil : pachchai pookosu

Parts used : whole plant

Chemical constituents:

Broccoli is high in vitamin C, as well as dietary fiber; it also contains multiple nutrients with potent anti-cancer properties, such as di-indolylmethane and small amounts of selenium. It also contains the compound glucoraphanin, which can be processed into an anti-cancer compound sulforaphane, and an excellent source of indole-3-carbinol, a chemical which boosts DNA repair in cells and appears to block the growth of cancer cells. Broccoli has the highest levels of carotenoids in the brassica family. It is particularly rich in lutein and also provides a modest amount of beta-carotene.

Uses:

It is a potent modulator of the innate immune response system with anti-viral, anti-bacterial and anti-cancer activity. Indole-3-carbinol, a chemical which present in boosts DNA repair in cells and appears to block the growth of cancer cells. A high intake of broccoli has been found to reduce the risk of aggressive prostate cancer. Broccoli consumption has also been shown to be beneficial in the prevention of heart disease.

PLANT PROFILE-2

Fig: 4 Spinacia oleracea

Description of plant: Spinach

Botanical information:

Botanical name : Spinacia oleracea.

Family : Amaranthaceae, formerly Chenopodiaceae Mimosaceae

Habitat : Asia, probably Persia

Vernacular names:

Hindi : Palak

Marathi : Palak

Malayalam : cheera, bayala

Sanskrit : Palakya

Tamil : Pasalakeerai

Parts used : Leaves

Chemical constituents:

Spinach leaves, containing several active components, including flavonoids, exhibit antioxidative, antiproliferative, and anti-inflammatory properties in biological systems. A powerful, water-soluble, natural antioxidant mixture (NAO), which specifically inhibits the lipoxygenase enzyme, was isolated from spinach leaves. Spinach contains an impressive range of active substances which are indispensable to the body. In its structure we find mineral salts (sodium, potassium, calcium, phosphorus, magnesium, sulfur, iron, zinc, manganese, and iodine, copper), vitamins (C, B1, B2, B6, PP, E, K, folic acid, vitamin A), chlorophyll, amino acids such as arginine and lysine, but also lipids, and fibers. With only 17 calories, 100 grams of spinach supplies the body with 5 mg of iron, 500 mg of potassium, 170 mg of calcium, 23 mg of vitamin C, 2 mg of vitamin E, 150 micrograms of folic acid, 3500 micrograms of beta-carotene and 7 grams of alimentary fibers.

Uses:

The plant is used against throat and lung affections.

AIM AND OBJECTIVE

The use of All-trans retinoic acid in health prospects is growing enormous, a study to investigate the estimation of the All-trans retinoic acid which is widely used as an Anti-oxidant, in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract has been proposed. There is no validated RP-HPLC method reported for the estimation of All-trans retinoic acid present in the plant Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea).

Hence the present study was aimed to

- Develop a new RP- HPLC method for the estimation of All-trans retinoic acid in different extract of these plants.
- Validate the developed method as per ICH guidelines.
- And to quantify All-trans retinoic acid in extract of these plants and dried leaves of the same.

For the simultaneous estimation of All-trans retinoic acid in extracts HPLC, HPTLC and UV-Spectroscopic methods are considered to be the most suitable. These methods are powerful, extremely precise, accurate, sensitive, specific, linear and rapid in analyzing the sample.

For the estimation of the drugs present in the extracts, RP-HPLC method is considered to be more suitable since this is a powerful and rugged method. In this study, HPLC with PDA detector was used the estimation of All-trans retinoic acid in Broccoli and Spinach**.**

MATERIALS

Plant materials

Broccoli and Spinach was obtained from Hills area of Ooty, Tamil nadu.

Marker compounds

All-trans retinoic acid was purchased from Surien Pharmaceutical Pvt.Ltd Chennai (India). The purity of All Trans retinoic acid was reported to be 99.6% w/w.

Chemicals and Solvents

Acetonitrile HPLC grade	–	Merck specialties, Mumbai.
Ortho phosphoric acid (Analytical grade)	–	SD fine chemicals, Mumbai.
Distilled water	–	Merck specialties, Mumbai.

Instruments

- HPLC system (Shimadzu, Japan) with LCsolution software.

- Elico pH meter LI 127.
- Shimadzu LC-20 AT HPLC.

  System : Shimadzu gradient HPLC

  Pump : LC – 20AT prominence solvent delivery system

  Detector : SPD-M20A Prominence Diode array detector

  Injector : Rheodyne 7725i with 20 µl loop

- Shimadzu 1600 LC-UV Spectrophotometer.
- Solvent filtration unit – Millipore.
- Shimadzu electronic balance AY 220.
- Ultra Cooling centrifuge – Remi, IndiaPerkin elmer Lambda 25 UV/Vis spectrophotometer with win lab software.
- Branson 1510 sonicator.
- Solvent filtration unit – Millipore.
- Mettler AT 200 weighing balance.
- Prama rotatory evaporator

METHODOLOGY

Selection of wavelength

Accurately weighed (1 mg) of All-trans retinoic acid was transferred in to a separate volumetric flask and dissolved in 10 ml methanol to produce 100 µg/ml of stock solution. A working standard solution containing 10µg/ml was prepared and the UV spectrum was recorded by scanning in the range of 200 nm to 400 nm. The chromatographic conditions used for the initial run is given below,

- ✓ **Chromatographic conditions-1**

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A: hexane sulphonic acid pH 3.5

Solvent B: methanol

Solvent ratio : 50:50

Detection : 215 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : water

Column temperature : room temperature (20ºC)

- ✓ **Chromatographic conditions-2**

Stationary phase : Phenomenex C18 column

Mobile phase : Solvent A: potassium dihydrogen orthophosphate

20mM pH 3.5, Solvent B: acetonitrile

Solvent ratio : 60:40

Detection : 261 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : water

Column temperature : room temperature (20ºC)

- ✓ **Chromatographic conditions-3**

Stationary phase : Phenomenex C18 Column

Mobile phase : Solvent A: Water

Solvent B: acetonitrile

Solvent ratio : 60:40

Detection : 220 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : water

Column temperature : room temperature (20ºC)

- ✓ **Chromatographic conditions-4**

Stationary phase : Phenomenex C18 Column

Mobile phase : Solvent A: ortho phosphoric acid pH-4

Solvent B: acetonitrile: methanol (50:50)

Solvent ratio : 55:45

Detection : 201 nm

Flow rate : 1.0 ml/min

Sample size : 20 µl

Needle wash : water

Column temperature : room temperature (20ºC)

While developing RP-HPLC method with different mobile phases like acetonitrile**:** methanol, acetonitrile**:** buffer (acetate, phosphate buffer), and methanol**:** buffer was used, but no favourable results obtained. But the mobile phase consisting of ortho phosphoric acid and acetonitrile in the ratio 55:45 gave acceptable peak.

Hence, further modification was made in the mobile phase to get a sharp symmetrical peak with good resolution.

4) OPTIMIZATION OF SEPARATION CONDITIONS

- **Effect of ratio of mobile phase**

The mobile phase of ortho phosphoric acid and acetonitrile in various ratios, 50:55, 55:45, 60:40, 70:30, were tried and the chromatograms were recorded at 201 nm with a flow rate of 1ml/min. At the ratio of 55:45 of ortho phosphoric acid and acetonitrile was selected as the ideal ratio for the estimation of All-trans retinoic acid.

- **Effect of flow rate:**

Keeping the ortho phosphoric acid and acetonitrile mobile phase ratio at 55:45 chromatograms were recorded at a flow rate of 1ml/min. At this flow rate, the peaks were sharp with good resolution. So 1ml/min was kept constant for the analysis (flow rate 1.0ml/min 1.1ml/min, up to 1.5ml/min were also tried, but did not give any satisfactory results).

Fixed chromatographic conditions:

Based on the above studies, the following chromatographic conditions were finally optimized for the simultaneous estimation of All-trans retinoic acid in Broccoli (flower head) and Spinach (leaf) extract**.**

Stationary phase	: Phenomenex C18 column
Mobile phase	: Solvent A: ortho phosphoric acid pH4
	Solvent B: acetonitrile
Solvent ratio	: 55:45
Detection	: 201 nm
Flow rate	: 1.0 ml/min

Sample size	: 20 µl
Needle wash	: water
Column temperature	: room temperature (20ºC)

SELECTION OF EXTRACTION PROCEDURE FOR THE PLANTS:

EXTRACTION PROCEDUREOF BROCCOLI

Method I.

Weighed accurately about 10gm of sample(broccoli) in a beaker, then 30ml of hexane was added and stored in room temperature for two days. Filter the sample solution and take 10 ml of filtrate (hexane layer), to this 30ml of di-chloride methane was added and kept for 2 days in room temperature. finally separated the two layers.

Method II.

To the weighed sample of broccoli (10gm), 20ml of chloroform was added and Stored at room temperature for two days. Filter the sample solution and collected the filtrate.

Method III.

10 gms of broccoli flowers was homogenized in a analytical grinder, added 50 ml of pure water and mixed allowed to autolyze for 2hrs at 25˚C to form a paste. This paste was extracted with ethyl acetate which were combined and dried at 35˚C under vacuum rotary evaporator. This residue was dissolved in 50ml of 10% ethanolic in water and washed 3 times with equal volume hexane to remove the Non-polar contaminants. Now this Ethyl acetate extract was washed with equal volume of ethyl acetate. This Ethyl acetate extract layer was pooled over anhydrous sodium sulphate and filtered through a 0.45µm membrane; filtrate was dried at 35˚C under vacuum in a rotary evaporator. This residue was dissolved in measured volume of methanol solvent.

EXTRACTIONPROCEDUREFOR SPINACH:

Method I.

Accurately weighed sample of spinach was shaken with dry ether for 3 hours at room temperature. Collected residue was then extracted with two successive lots of dry acetone. Combine the filtrate of ether and acetone extracts and removed the solvent by evaporation.

Method II.

Weighed sample of spinach was shaken with toluene at room temperature. Then collected residue was extracted with two successive amount of methanol. Combine the extracts after filtration and removed the solvent by evaporation.

Method III.

Weigh about 0.5 – 1.0 g of fresh spinach leaves (avoid using stems or thick veins). Cut or tear the spinach leaves into small pieces and placed in a mortar along with 2.0 ml of acetone. Grind with a pestle until the spinach leaves have been broken into particles. Using a Pasteur pipette or spatula, transfer the mixture to a centrifuge tube. Rinse the mortar and pestle with 2.0 ml of cold acetone, and transferred the remaining mixture to the centrifuge tube. Cap tightly. Added 2.0 ml of hexane to the centrifuge tube, and shaken the mixture thoroughly. The separated hexane layer (dark green pigment layer) is transferred into a clean test tube. Traces of water remained in the solution is removed before separating the components through chromatography by the addition of 0.5g of anhydrous sodium sulphate. After standing for 5 minutes, transfer the liquid into another clean test tube.

QUANTIFICATION AND VALIDATION

Preparation of standard stock solutions

Accurately weighed 10 mg of All-trans retinoic acid, transferred into a 10 ml volumetric flask, dissolved in 10ml of methanol, to produce 1 mg/ml stock solution.

Preparation of working standard stock solutions

Calibration curves

Appropriate aliquots from the standard solutions were transferred to a series of 10 ml volumetric flasks, and the volume in each flask was adjusted to the mark with Mobile phase. The resulting concentrations of All-trans retinoic acid in the flasks ranged from 10-50 µg/ml.

PREPARATION OF PLANT EXTRACTS

Preparation of sample solution

10 mg of plant extract was weighed accurately and transferred into china dish to remove hexane by evaporation and make up to 10ml in volumetric flask, with mobile phase. All the above solutions were filtered through 0.45 µm nylon filter paper prior to use.

Recording of chromatogram

With the optimized chromatographic conditions mentioned above, perform a steady baseline for about 20 min. After the stabilization of the baseline for about 20 min, the standard solution were injected and chromatograms were recorded until the reproducibility of the peak areas were found satisfactory and finally 10 µg/ml of the standard solution of the All-trans retinoic acid and Standard solutions containing 10-50µg/ml of same standard were injected and chromatograms were recorded. .Retention time of All-trans retinoic acid was found to be 8.8 shown in **Fig. 5, 6, 7, 8 and 9** respectively.

The procedure was repeated using for the sample (extract) solutions and the chromatograms were shown in **Fig.11, 12, 13, 14, 15, 16.** Peak areas of the sample chromatograms were recorded and the amount of All-trans retinoic acid was calculated from the regression equation.

VALIDATION OF THE HPLC METHOD

The method was validated for linearity, accuracy, precision, robustness, sensitivity, system suitability, LOD and LOQ as per ICH guidelines (1996) by the following procedures

System suitability

System suitability of method was performed by six replicate analysis of the system suitability solution of All-trans retinoic acid. The acceptance criterion was ±2% for the percentage relative standard deviation (%RSD) of peak area and retention time. The resolution and number of theoretical plates and HETP were also determined.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. Peak purity of the method was evaluated by calculating the difference between peak angle (θ_p) and peak threshold angle (θ_{th}). If $\theta_p<\theta_{th}$, the peak is considered spectrally homogeneous; otherwise the peak is influenced by the presence (i.e., additional absorption) of another substance. Peak purity was assessed by using LC Solution software for the HPLC/PDA system based on the degree of similarity of UV spectra across the peak in the range of 190-800 nm. The separation was performed on a Phenomenex C18 column (particle size 5 µm; 250×4.6 mm, id). Peak purity evaluation was performed with the objective of obtaining additional supportive information during the selection of appropriate analytical conditions that allowed specific determination of All-trans retinoic acid.

Linearity (Calibration curve)

Linearity was performed for All-trans retinoic acid standard solutions in the concentration range of 10-50 µg/ml. Calibration curves were constructed by plotting peak areas against analyte concentrations. The linearity was assessed by calculating the slope, y-intercept and coefficient of determination (r^2) using Microsoft Excel 2007 program.

Precision

The precision of the method was examined by performing the intra-day and inter-day assays of six replicate injections of the standard solution. The intra-day assay precision test was performed at intervals of 4 h in 1 day, while the inter-day assay precision test was performed over 6 days.

Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ of the analyte was determined by k SD/b where k is a constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and b is the slope of the concentration/response graph.

Accuracy

The accuracy of the method was determined by calculating the recoveries of All-trans retinoic acid by the method of standard addition. Known amount of the standards (80, 100 and 120%) were spiked in to the pre-analyzed sample solutions, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curves.

RESULTS AND DISCUSSION

HPLC METHOD

A RP-HPLC method was developed for the estimation of All-trans retinoic acid in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract. The chromatographic conditions were stabilized in order to provide a good performance of the assay. The standard and sample solutions were prepared and chromatograms were recorded. This project proposes a method for the determination of All-trans retinoic acid in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract.

Method validation

The method was developed for analyzing All-trans retinoic and validated for linearity, accuracy, precision, LOD, LOQ, robustness and system suitability as per ICH guidelines.

System suitability

Six replicate injections of the system suitability solution gave %RSD values for retention time and peak area within 2%, indicating low variation of the measured values (Table 6). The symmetry of all peaks was < 2. The resolution (R) of All-trans retinoic acid, indicating a high degree of peak separation (R>2). The efficiency of the column, as expressed by the number of theoretical plates, was more than 2000. These results indicate the suitability of the RP-HPLC system and conditions of the developed method.

Calibration curve (Linearity)

Five working standard solutions of All-trans retinoic acid in the range of 10-50 µg/ml were prepared respectively. Each solution was injected in six replicate and the linear regressions analysis of All-trans retinoic acid were constructed by plotting the peak area of the analyte (y) versus analyte concentration (µg /ml) on (x) axis**.** The slopes, y-intercepts and correlation coefficients (r^2) obtained from regression analysis are shown in Table 3, Figure 23. The regression equation was y=8315.1x+5040.1(r^2 =0.999) for All-trans retinoic acid.

Precision

The intra-day and inter-day precision result for All-trans retinoic acids at the three concentrations are presented in Table 4, 5. Intra-day assay precision was performed at an interval of 4 h, whereas inter-day assay precision was performed over 6 days for All-trans Retinoic acid. It was shown that the %RSD values for retention time of analyte was< 1%,

while the %RSD values for peak area of analyte was< 2%. The low values of %RSD (<2%) reflected the high precision of the method.

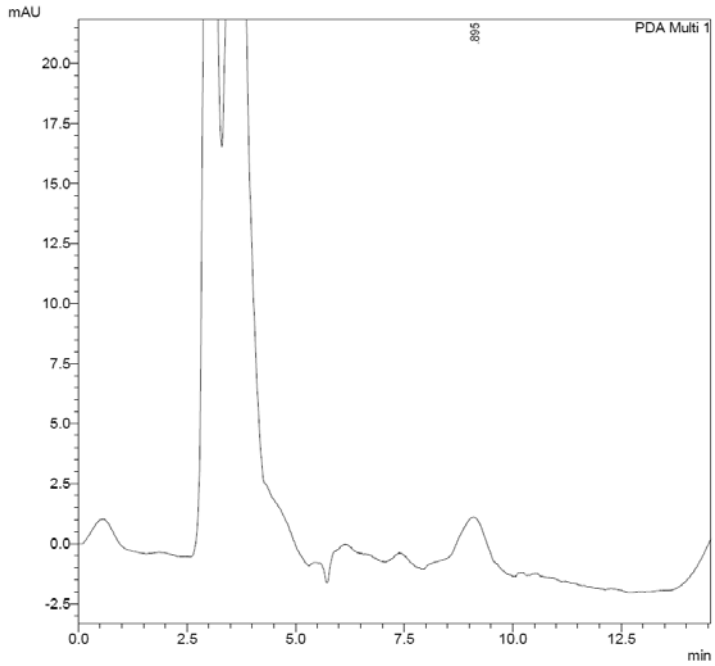
Accuracy

As shown in Table5, the recovery of the investigated components ranged from 100.37-100.94 and their %RSD values were all < 2%. It was known from recovery tests that all percentage recoveries were within 98-102%, indicating the good accuracy of the developed method.

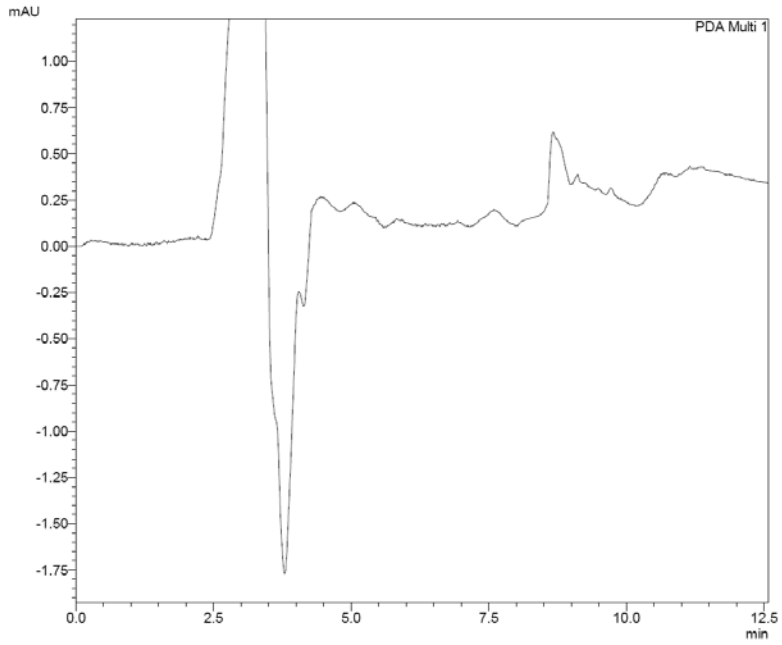
Limit of detection and Limit of quantification

The LOD was found to be 40µg/ml and the LOQ was found to be 120 µg/ml for All-trans retinoic acid (Table no: 6). These results indicated that the method provided adequate sensitivity.

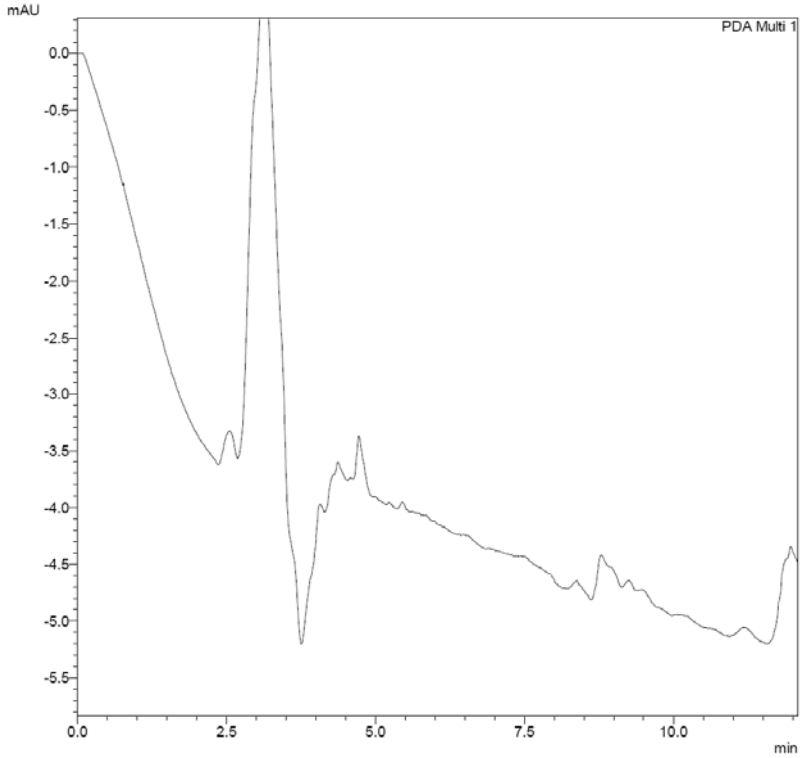
SELECTION OF EXTRACT



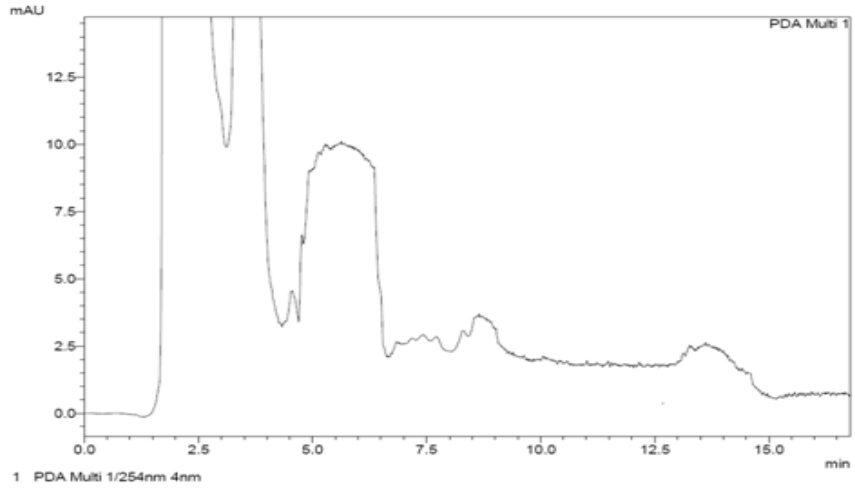
Trial chromatogram of Broccoli extraction method I procedure



Trail chromatogram of Broccoli method II procedure



Trail chromatogram of Spinach method I procedure



Trail chromatogram of Spinach method II procedure

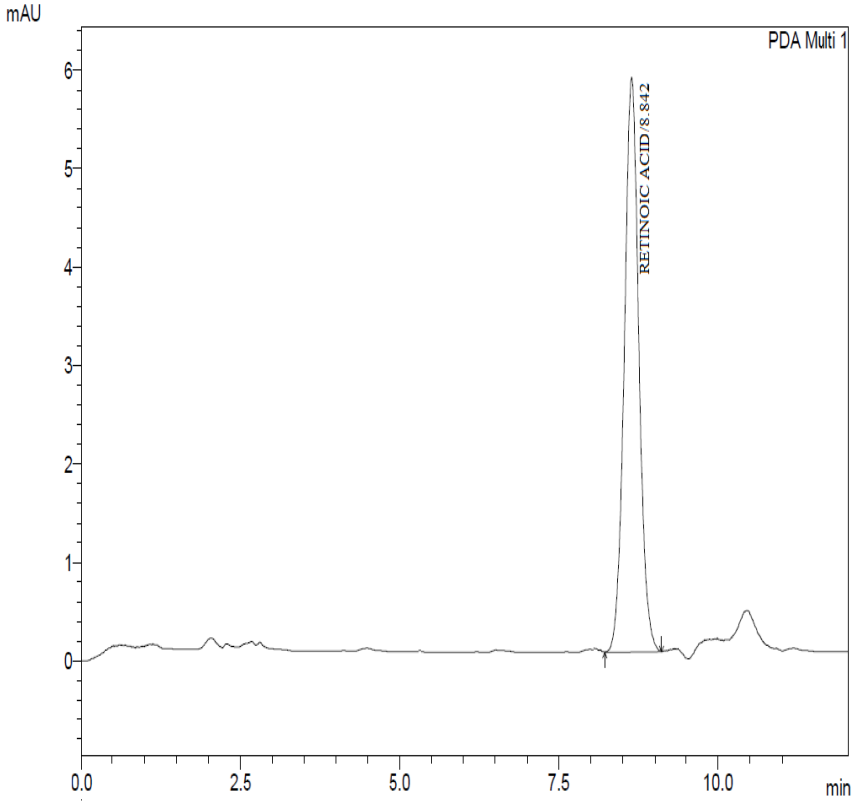


Fig: 5 Standard chromatogram of All-trans retinoic acid standard (10µg/ml)

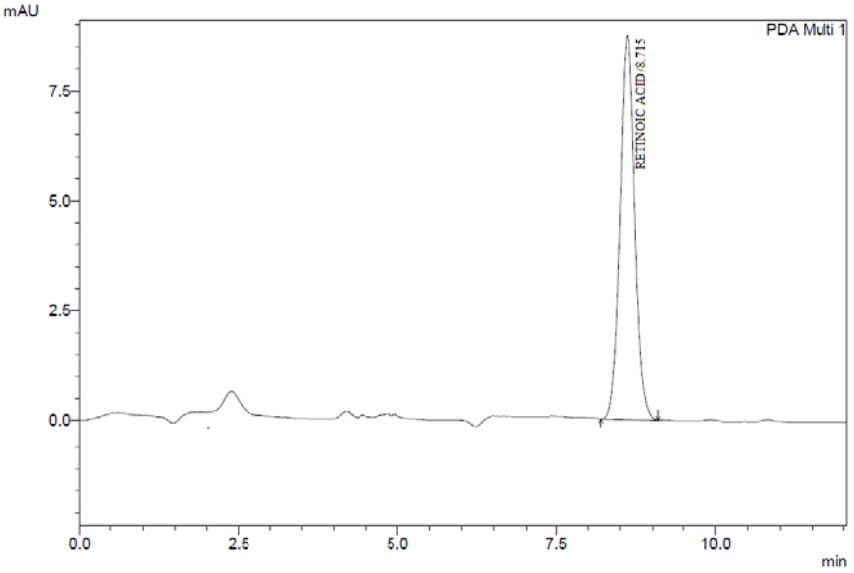


Fig: 6 Standard chromatogram of All-trans retinoic acid standard (20µg/ml)

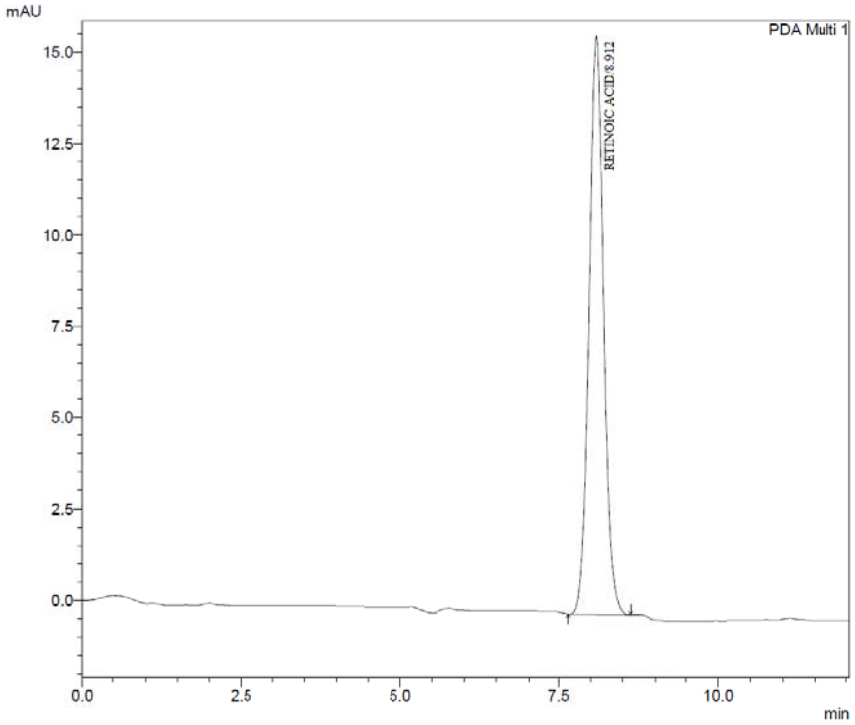


Fig: 7 Standard chromatogram of All-trans retinoic acid standard (30µg/ml)

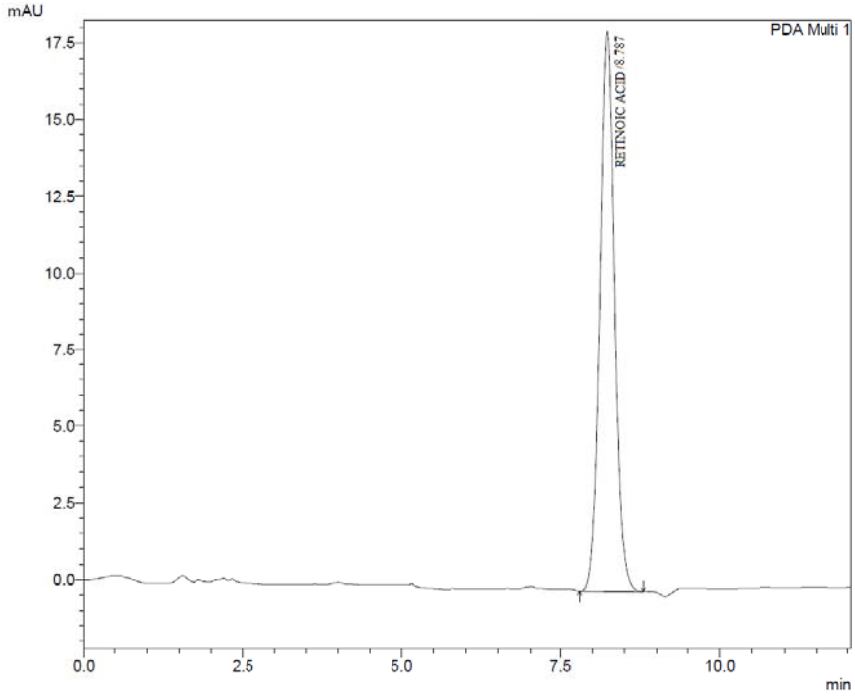


Fig: 8 Standard chromatogram of All-trans retinoic acid standard (40µg/ml)

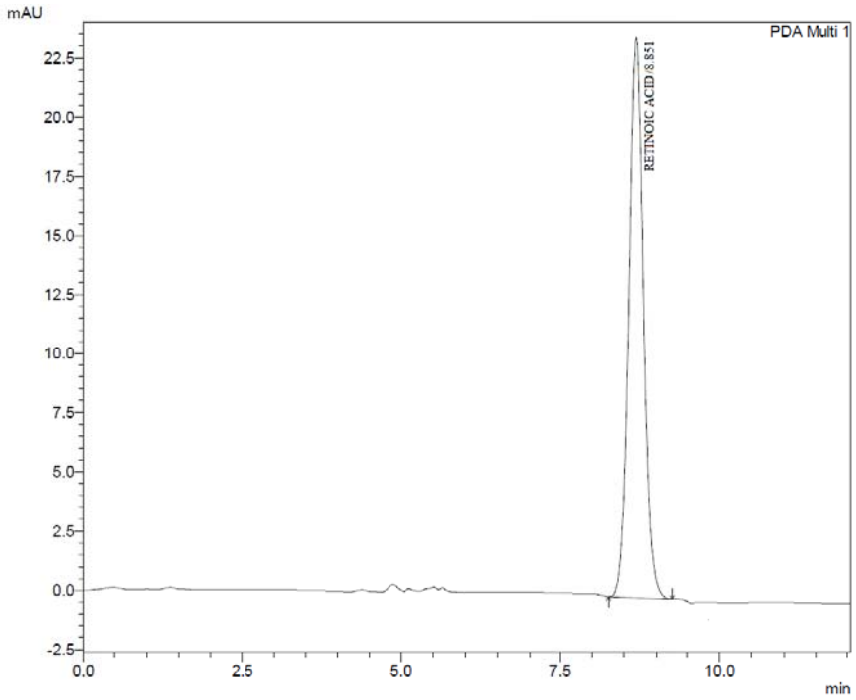


Fig: 9 Standard chromatogram of All-trans retinoic acid standard (50µg/ml)

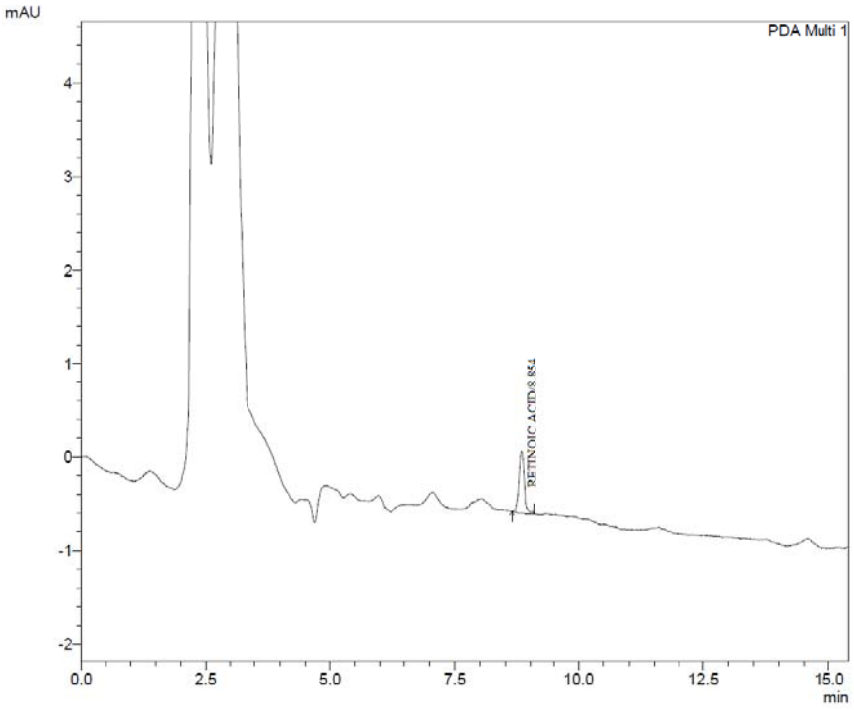


Fig: 10 Chromatogram of Broccoli (flower head) extract (35days)

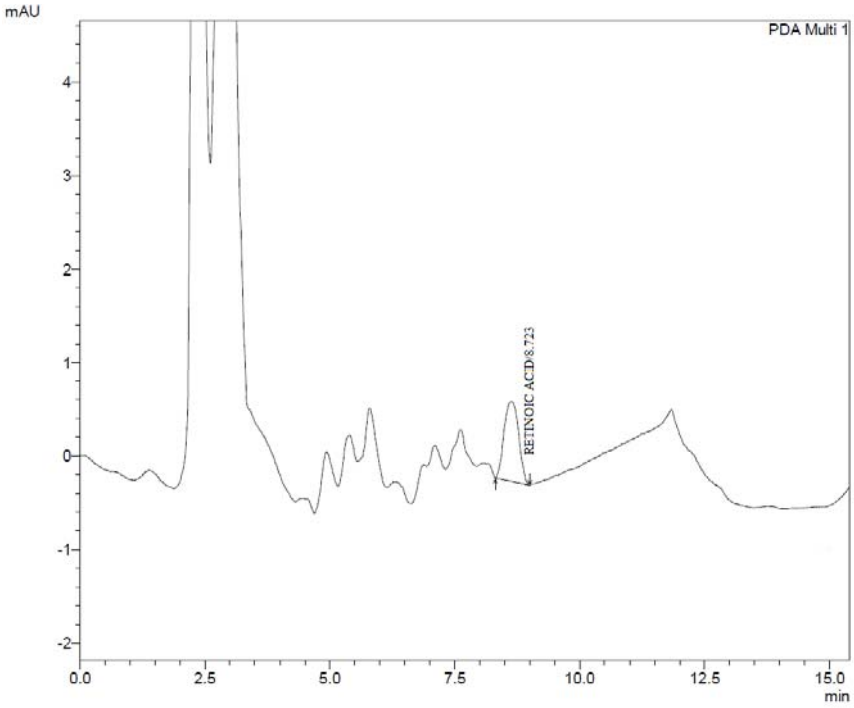


Fig: 11 Chromatogram of Broccoli (flower head) extract (40days)

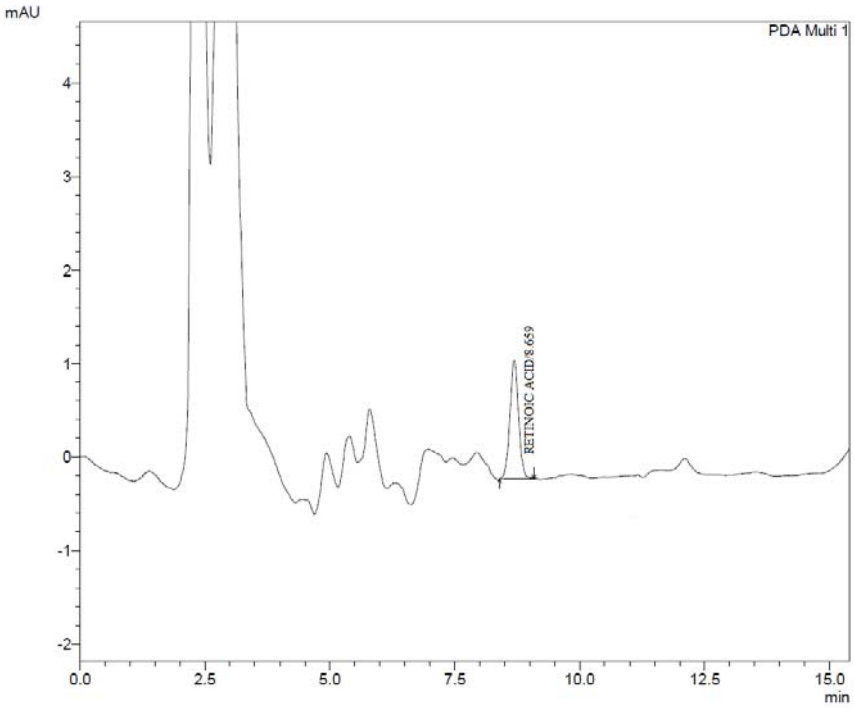


Fig: 12 Chromatogram of Broccoli (flower head) extract (45days)

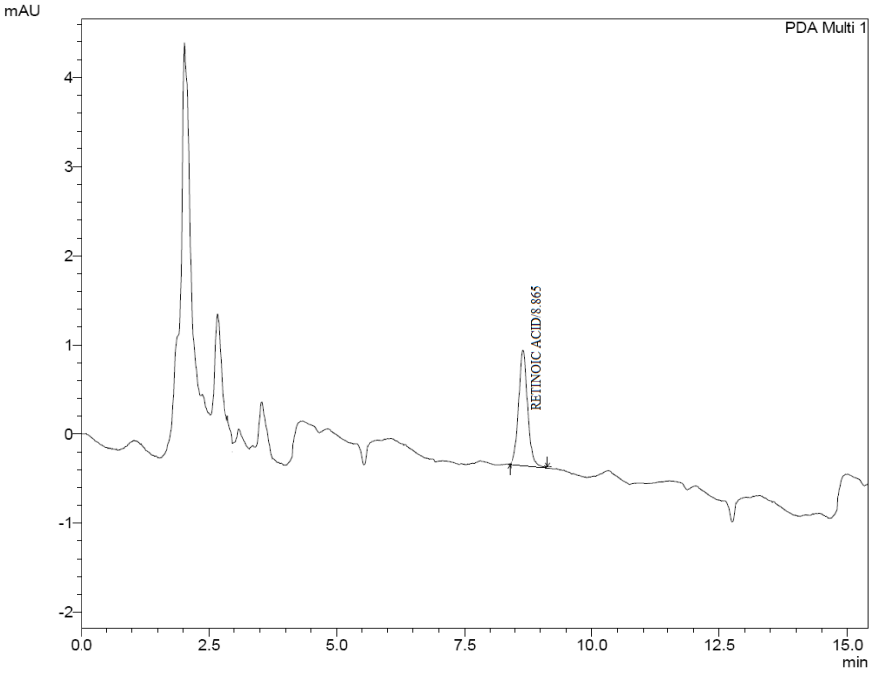


Fig: 13Chromatogram of Spinach leaf extract (20 days)

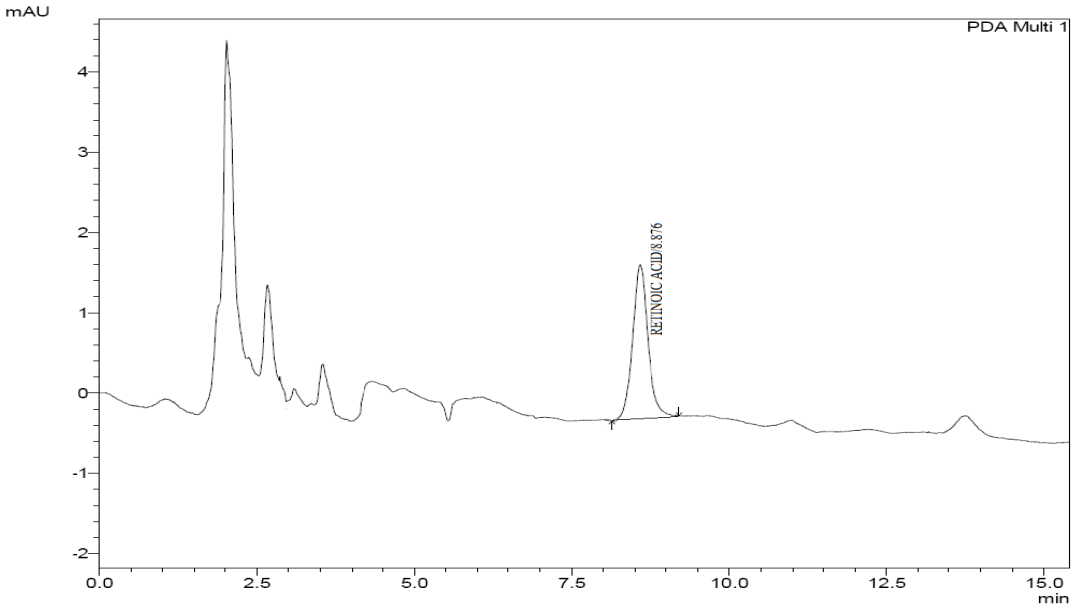


Fig: 14 Chromatogram of Spinach leaf extract (25 days)

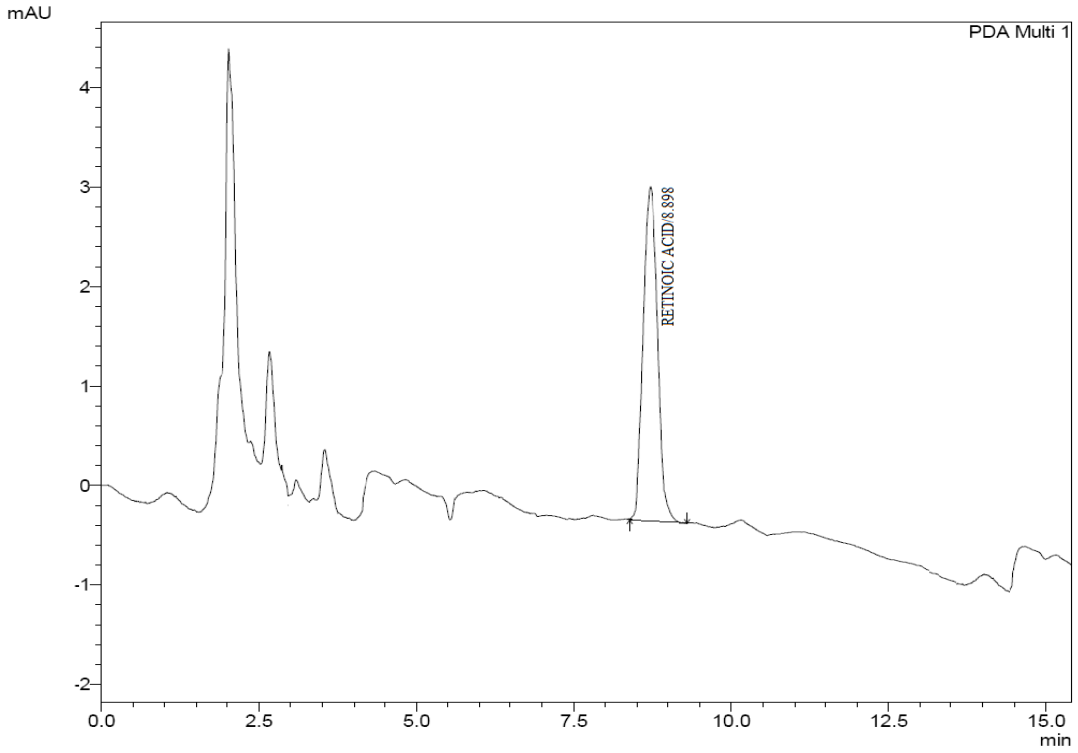


Fig: 15 Chromatogram of Spinach leaf extract (30 days)

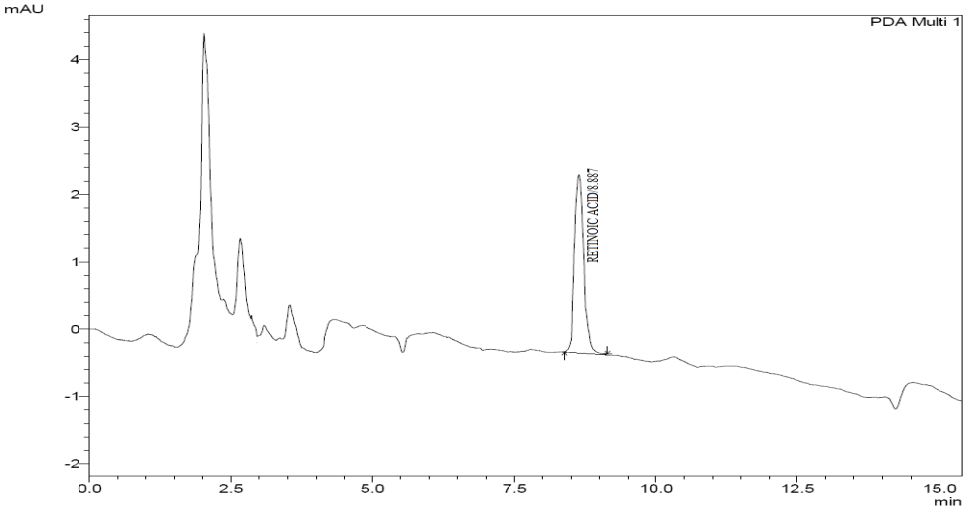


Fig: 16 Chromatogram of Spinach leaf extract (dried leaves)

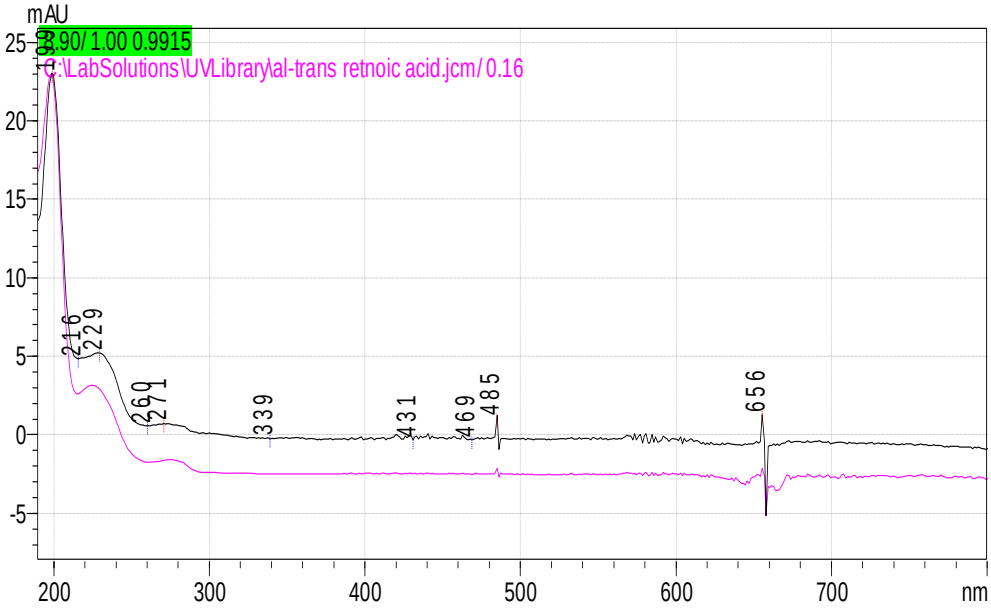


Fig: 17 UV spectrum of All-trans retinoic acid standard solution

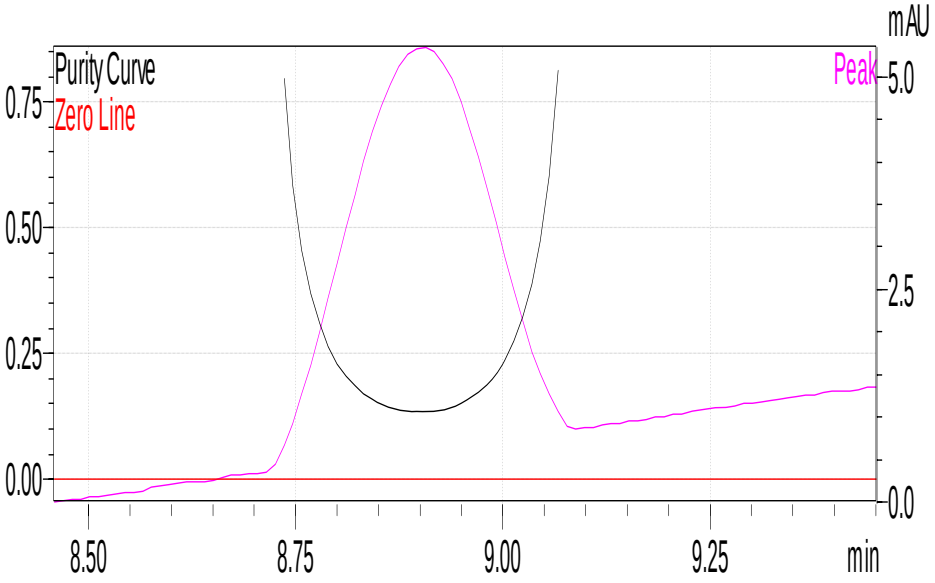


Fig: 18 purity profile of All-trans of retinoic acid (10 µg/ml)

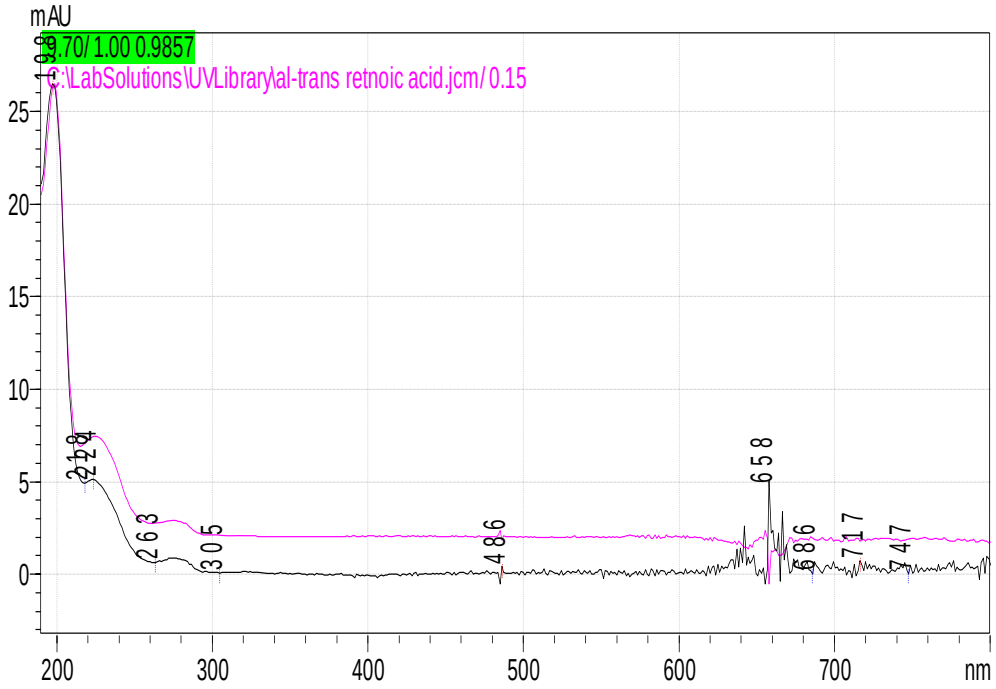


Fig: 19 UV spectrum of Broccoli (flower head) extract

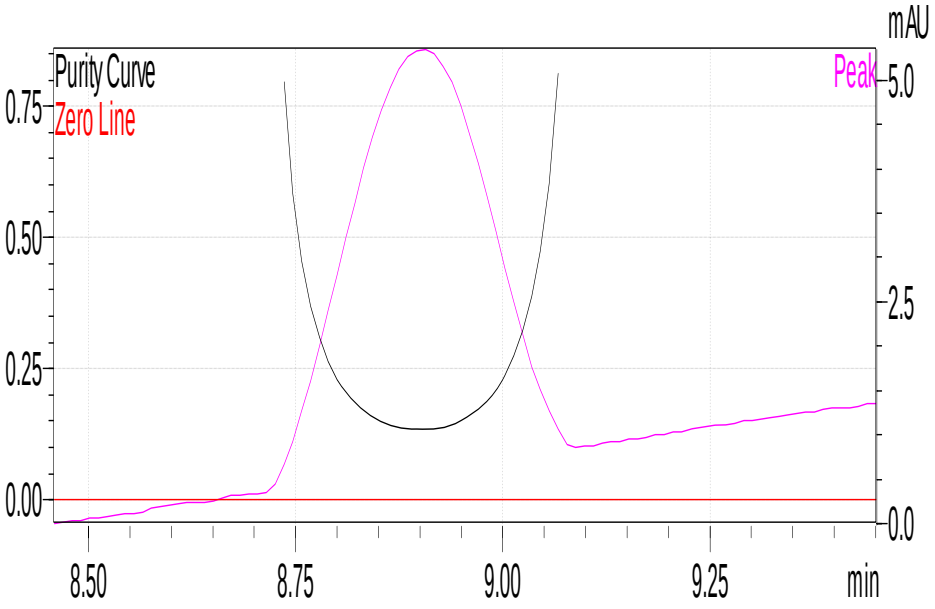


Fig: 20 purity profile of Broccoli flower head extract

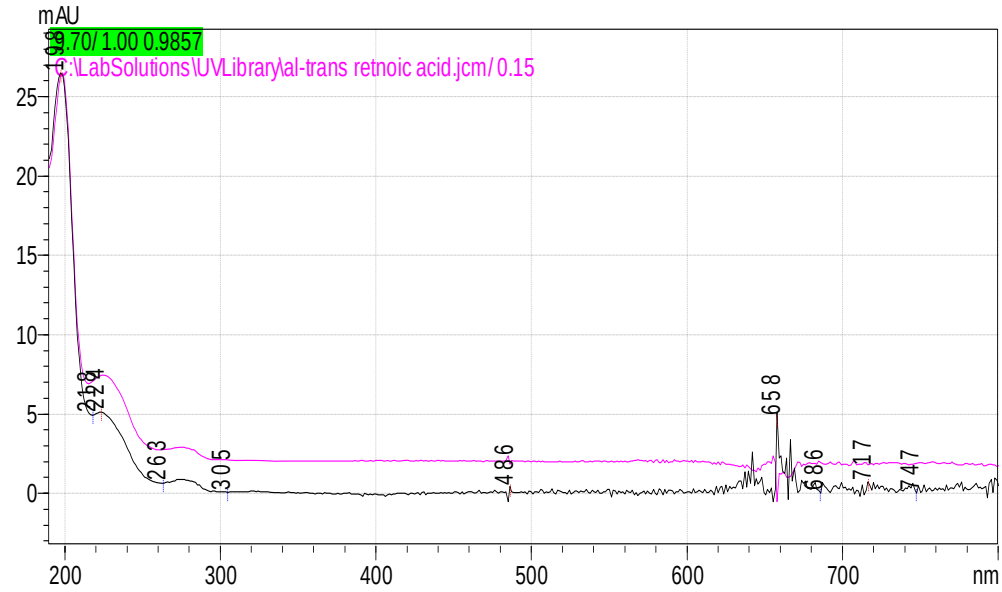


Fig: 21 UV spectrum of Spinach leaf extract

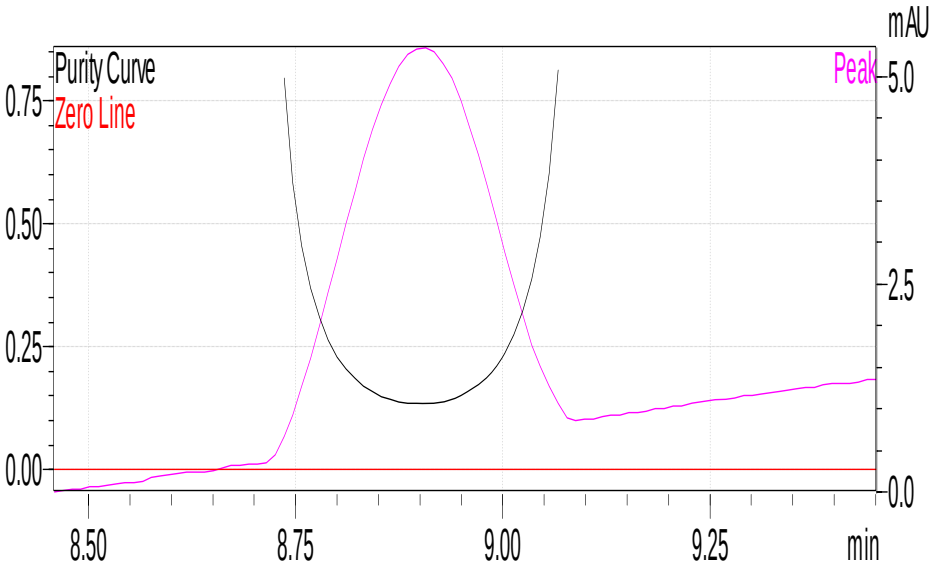


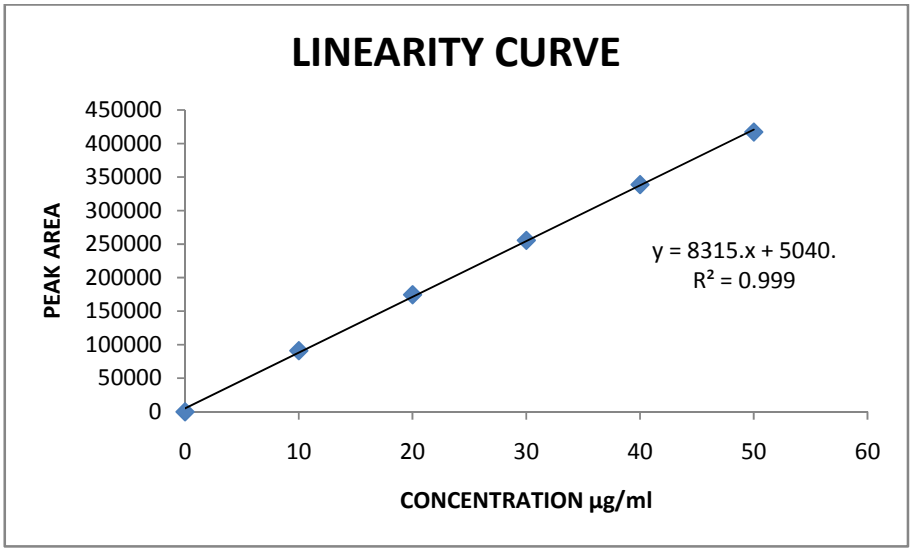
Fig: 22 purity profile of Spinach leaf extract

LINEARITY RANGE OF ALL-TRANS RETINOIC ACID

Table: 3 Calibration standard peak area

S.NO	Con of drug(µg/ml)	peak area
1	10	91176
2	20	174576
3	30	255657
4	40	338880
5	50	417220

Fig: 23 Linearity curve of All-trans retinoic acid



Accuracy and Precision Studies (intraday)

Table no: 4 Accuracy and Precision Studies (Intra - Day)

S.No	Conc of drug(µg/ml)	Mean peak area	%Accuracy	%RSD
1	10	91156	101.01	0.86
2	20	174536	101.04	0.87
3	30	254657	100.24	0.88
4	40	337680	100.18	0.85
5	50	417220	99.30	0.81

Table no: 5 Accuracy precision studies(inter-day)

S.No	Conc. of drug(µg/ml)	Mean peak area	%Accuracy	%RSD
1	10	91176	100.82	0.85
2	20	174576	100.94	0.86
3	30	255657	100.46	0.85
4	40	338880	100.37	0.84
5	50	417220	99.14	0.83

Table no: 6 System suitabilities studies

S.NO	PARAMETERS	All-trans retinoic acid
1	Theoritical plate	7162.234
2	HETP	28.943
3	Tailing factor	0.953
4	LOD(µg/ml)	40
5	LOQ(µg/ml)	120
6	Resolution	1.206
7	K	0.952

Recovery study of All-trans retinoic acid

Table no:7 Recovery studies

Components	Quantity added %	Total quantity present, µg/ml	Quantity found, µg/ml	Recovery, %	%RSD
All-trans retinoic acid	0	9.669	9.435	97.52	0.712
	80	17.844	17.669	98.97	0.642
	100	18.938	18.838	97.88	0.768
	120	20.831	20.455	98.19	1.664

Table no: 8 Ruggedness studies

Marker compound	Name of analyst	Concentration	Mean peak area	%RSD
All-trans retinoic acid	Day-1 Analyst-1	10µg/ml	91176	0.85
	Day-1 Analyst-2	10µg/ml	91181	0.87

Quantification studies:

Quantitation of All-trans retinoic acid in plant extracts:

Table: 9a Quantification studies

Components	No. of weeks	Ethyl acetate flower head extract of Broccoli mg/g
All-trans retinoic acid	5th	0.04
	51/2	0.07
	6th	0.08

Table:9b Quantification studies

Components	No. of weeks	Hexane leaf extract of Spinach mg/g
All-trans retinoic acid	3rd	0.170
	31/2	0.250
	4th	0.370
	Dried leaves	0.364

SUMMARY AND CONCLUSION

The developed RP-HPLC method allows rapid and precise determination of All-trans retinoic acid content in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract with an economical mobile phase. All-trans retinoic acid was higher in 6^{th} week crop (0.08mg) of Broccoli (Brassica oleracea) and 4^{th} week crop (0.370mg) of Spinach (Spinacia oleracea).

The scope of the present work is to expand the optimization of the chromatographic conditions, to develop RP-HPLC method, a series of mobile phases were tried, among the various mobile phases ortho phosphoric acid and acetonitrile mixture (55:45) was found to be an ideal mobile phase, since it gave a good resolution and peak shapes with perfect optimization. The flow rate was optimized at 1 ml/min.

The Linearity and correlation coefficient of All-trans retinoic acid was found to be 10-50 µg/ml 0.9998 respectively.

The limit of detection for All-trans retinoic acid was found to be 40 µg/ml and the limit of quantification was found to be 120 µg/ml.

The method was known to be accurate with %recoveries ranging from 97.52 to 98.97%.

The accuracy and precision study was performed at two levels intra-day and inter-day. The developed method showed good accuracy and precision. The inter-day accuracy ranges between 99.14-100.94% with %RSD between 0.83-0.86. The intra-day accuracy range between 99.30-100.24% with %RSD between 0.81-0.87.

The isocratic elution technique developed for the determination of All-trans retinoic acid in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract is ideally suited for rapid and routine analysis. This method shows good reproducibility of the results. Furthermore this method was simple, sensitive, accurate and can be applied to all kinds of plant extract.

CONCULSION

The RP-HPLC method for analysis of All-trans retinoic acid in Broccoli (Brassica oleracea) and Spinach (Spinacia oleracea) extract was found to be accurate and precise. The proposed method was validated according to ICH guidelines and correlating the obtained values with the standard values, satisfactory results were obtained.

Since the Anti oxidant Compound have been of interest in health benefits, this Project can be useful for further research of All-trans retinoic acid in other plant extracts also.

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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ALL-TRANS RETINOIC ACID IN PLANT EXTRACT OF BROCCOLI AND SPINACH.

Abstract

A simple high-performance liquid chromatography method using a photo diode array detector (PDA) is developed for the estimation of All-trans retinoic acid in plant extract of Broccoli and Spinach. The optimal analytical conditions are investigated to obtain the best resolution and the highest UV sensitivity for the quantitative detection of All-trans retinoic acid. The optimized conditions (acetonitrile : ortho phosphoric acid buffer, pH 4, isocratic elution system on a C18 reversed-phase column with a flow rate of 1 ml/min and UV absorbance at 201 nm) allowed a specific and repeatable separation of the studied analyte to be achieved. Elution is successfully achieved within 9 min. Calibration curves are linear in 10-50 µ/ml range. The limit of detection and limit of quantitation limit values were found to be 40µ/ml and 120µl respectively. The validated method is applied to the determination of the specific phyto chemical markers All-trans retinoic acid in Broccoli and Spinach extracts. The recovery values ranged between 97.52 to 98.97%. The described HPLC method appears suitable for the differentiation and determination of the All-trans retinoic acid and can be considered an effective and alternative procedure for the analyses of this important class of natural compounds.